Identification of Coeliac Disease Triggering Glutenin Peptides and Their Measurement in Foods

Donnelly, Suzanne Claire

Awarding institution:
King's College London

Identification of Coeliac Disease Triggering Glutenin Peptides and Their Measurement in Foods

A thesis presented for the degree of Doctor of Philosophy

by

Dr Suzanne Claire Donnelly

BSc (Med Sci), MBChB, MRCP

King's College London,

Division of Diabetes and Nutritional Sciences,

The Rayne Institute,

University of London

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Abstract

Coeliac disease is a common small intestinal disorder affecting approximately 1% of the Northern European population. It is caused by an inappropriate immune response to dietary gluten found in wheat, rye, barley and, for a small minority of patients, oats. There is mounting evidence to suggest that the immune response of coeliac disease is not purely driven by gluten-sensitive T lymphocytes but also by an innate immune response mediated through interleukin-15.

Dietary gluten comprises of two major protein fractions, gliadins and glutenins. Considerable research has been carried out on the immunostimulatory components in wheat gliadin. Glutenins, newly discovered to be toxic to coeliac individuals, have limited evidence describing their immunostimulatory potential in coeliac disease. Glutenins can be further differentiated by their molecular weight into low and high molecular weight fractions. Small intestinal T-cell studies suggest that an *in vitro* response to certain high molecular weight glutenin proteins may occur in some, but not all, coeliac individuals. There is even less evidence available concerning the coeliac disease toxicity of low molecular weight glutenin proteins. The stimulating epitopes within these proteins are not fully understood.

This thesis describes the immunostimulatory potential of peptides contained in high molecular weight glutenins, glut 04 p721-735, and in low molecular weight glutenins, glt 156 p44-59, that have been implicated in a small number of coeliac individuals. The adaptive immune response to these peptides has been measured by proliferation assays using gluten-sensitive small intestinal lymphocytes and measurement of their interferon-γ secretion. The innate immune response has been assessed by morphometric measurement of small intestinal biopsies following overnight incubation with these peptides, followed by interleukin-15 assessment via secretion into the tissue culture supernatant.

A number of attempts have been made to raise monoclonal antibodies to these peptides. A variety of immunisation schedules have been attempted in host mice. A number of cell fusion experiments have been performed without success. The possible reasons for this lack of success are discussed.

Results generated by *in vitro* studies of coeliac small intestinal explants and isolated cells have improved our understanding of the pathogenesis of coeliac disease.

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List of abbreviations

AGA	Anti-gliadin antibodies
APC	Antigen-presenting Cell
ASM	Autologous serum medium
BCG	Bacille Calmette-Guérin
BMD	Bone mineral density
BSA	Bovine serum albumin
CD	Coeliac Disease
CFA	Complete Freund's adjuvant
CPM	Counts per minute
DXA	Dual-emission x-ray absorptiometry
DGP	Deamidated gliadin peptide
ECH	Enterocyte Cell Height
ECM	Extracellular Matrix
ELISA	Enzyme-linked immunosorbant assay
ELISPOT	Enzyme-linked immunosorbent spot
EMA	Anti-endomysial antibody
EATL	Enteropathy-associated T-cell lymphoma
DH	Dermatitis Herpetiformis
DM	Diabetes Mellitus
DMSO	Dimethyl sulphoxide
FCS	Foetal calf serum
FFIII	Frazer's Fraction III
GFD	Gluten Free Diet
GORD	Gastro-oesophageal reflux disease
GU	Gastric ulcer

GWAS	Genome-wide association study
^{3}H	Tritiated thymidine
H+E	Haematoxylin and eosin
HLA	Human Leukocyte Antigen
HMW	High molecular weight
HMWG	High molecular weight glutenin
HMW $glut_{04}$	High molecular weight glutenin 04 717-732
HRP	Horseradish peroxidase
IBS	Irritable bowel syndrome
IEL	Intra-epithelial lymphocytes
IFA	Incomplete Freund's adjuvant
IFN-γ	Interferon Gamma
IHD	Ischaemic heart disease
IL	Interleukin
ic	Intracutanous
im	Interamuscular
ip	Intraperitoneal
iv	Intravenous
KLH	Keyhole limpet haemocyanin
LMW	Low molecular weight
LMWG	Low molecular weight glutenin
LMW glt_{156}	Low molecular weight glutenin glt156 39-59
MAP	Multiple antigen peptide
MHC	Major Histocompatability complex
MMW	Medium molecular weight
mRNA	Messenger ribonucleic acid
NA	Not available

NHL	Non-Hodgkin's lymphoma
NRCD	Non-responsive coeliac disease
NK	Natural killer
OA	Osteoarthritis
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PCOS	Polycystic ovary syndrome
PHA	Phytohaemagglutinin
PPD	Purified protein derivative
PTG	Peptic-tryptic gliadin
PT gluten	Peptic-tryptic digest of industrial gluten
RA	Rheumatoid arthritis
RCD	Refractory coeliac disease
RPM	Revolutions per minute
RPMI	Rosewell Park Memorial Institute
SBBO	Small bowel bacterial overgrowth
sc	Subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
SI	Stimulation Index
SIL	Small intestinal lymphocytes
SLE	Systemic lupus erythematosus
T1DM	Type 1 diabetes mellitus
TCR	T-cell Receptor
TTd	Tetanus toxoid
tTG	Tissue Transglutaminase

Single letter code for amino acids

Amino Acid	Letter	Three-letter Code
ALANINE	A	ALA
CYSTEINE	C	CYS
ASPARTIC ACID	D	ASP
GLUTAMIC ACID	E	GLU
PHENYLALANINE	F	PHE
GLYCINE	G	GLY
HISTIDINE	H	HIS
ISOLEUCINE	I	ILE
LYSINE	K	LYS
LEUCINE	L	LEU
METHIONINE	M	MET
ASPARAGINE	N	ASP
PROLINE	P	PRO
GLUTAMINE	Q	GLN
ARGININE	R	ARG
SERINE	S	SER
THREONINE	T	THR
VALINE	V	VAL
TRYPTOPHAN	W	TRP
TYROSINE	Y	TYR

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Chapter 1: Introduction

Coeliac disease (CD) is also known as Coeliac Sprue or gluten-sensitive enteropathy. It is a common small intestinal disorder caused by an inappropriate immune response to dietary gluten. Ingestion of wheat, rye or barley proteins in susceptible individuals triggers an inflammatory response particularly in the proximal small intestinal mucosa causing damage to the villous architecture. In a small minority of patients this also happens with oat proteins. The disease manifests itself as a generalised malabsorption which typically presents as weight loss, diarrhoea or disorders associated with specific nutrient deficiencies such as anaemia or osteoporosis. However, since the advent of highly sensitive serological screening tests, less well defined forms of the disease are being increasingly recognised. Treatment is the life long, complete avoidance of gluten from the diet.

1.1 History of Coeliac Disease

One of the first descriptions of coeliac disease came from Areteus the Cappadocian who lived in the 2nd Century AD. He described the characteristic stool and noticed that it was more common in women than in men, and that children could also be affected (Adams 1856). However, one of the first modern documented descriptions of coeliac disease was a report written by the paediatrician, Samuel Gee in 1888 entitled "On the Coeliac Affection" after a lecture at Great Ormond Street Hospital. The report included a particularly elaborate description of the appearance of the stools from a patient suffering from "the coeliac affliction". Interestingly, the author speculated that the cause of the disease was probably diet-related. It was noted that if the diet was the cause however, this did not explain how in a family of children all raised the same way only one child would suffer from the disease. He observed the symptomatic improvement of a child who was "fed upon a quart of the best Dutch mussels daily" but who relapsed when the season for mussels was over. It is not clear whether this diet consisted solely of mussels, but presumably the child's improvement was as a result of the absence of dietary gluten, although this was not understood at the time. An avoidance of starch, amongst other foods, was recommended. In 1924, an American physician named Dr Hass recommended a diet restricting the use of carbohydrates, except ripe bananas, and fat, which was known as "the banana diet". This

diet was used extensively until the gluten-free diet was introduced. In Britain during the Second World War, coeliac children were allocated rations of dried bananas as a supplement. However, the cause was not realised until the early 1950s during the flour shortages in the Second World War when a Dutch physician, Dicke, noticed that the condition of many of his coeliac patients improved. His subsequent experiments lead to identification of wheat and related cereals as being the trigger for CD (Dicke 1953a and b).

1.2 Epidemiology

Early estimations of the prevalence of CD indicated that it was an uncommon disorder. This has now changed due to the advent of improved serological testing and endoscopic evaluation. Approximately 1% of the UK population has been reported (West 2003) where previously it was thought to affect 1:8000 in England and Wales in the 1950s (Fasano 2001). Coeliac disease has been found in many countries now across the world (Cook 2000, Gandolfi 2000, Gomez 2001, Hovell 2001, Imanzadeh 2005, Sood 2006, Ben Hariz 2007, Oliveira 2007, Abu-Zekry 2008, Mustalahi 2010, Wu 2010, Alarida 2011, Dalgic 2011, Xin-Qiong 20011). There is no data at present in sub-Saharan Africa although the highest prevalence of coeliac disease is to be found in the Sahrawi population of Arab-Berber origin living in Algeria (Catassi 1999). High levels of consanguinity, high frequencies of HLA-DQ2 and heavy gluten ingestion are potential explanations. Coeliac disease is known to be rare in Afro-Caribean individuals (Brar 2006).

1.3 Clinical Features of coeliac disease

1.3.1 Classical coeliac disease

The clinical manifestations of coeliac disease vary greatly and although this was perceived as a purely paediatric disease, the diagnosis is increasingly being made in adult life.

In adults, CD can manifest as a variety of symptoms presenting to almost any hospital department. The presenting symptoms may be varied including gastrointestinal, metabolic, neurological or psychological. The most common presenting gastrointestinal symptom is diarrhoea which can be continuous, intermittent or alternated with periods of constipation. It may be accompanied by weight loss, bloating, anorexia or abdominal pain. Isolated

increase in serum aminotransferase level caused by mild, non-progressive liver inflammation is also a common presentation (Rubio-Tapia 2007).

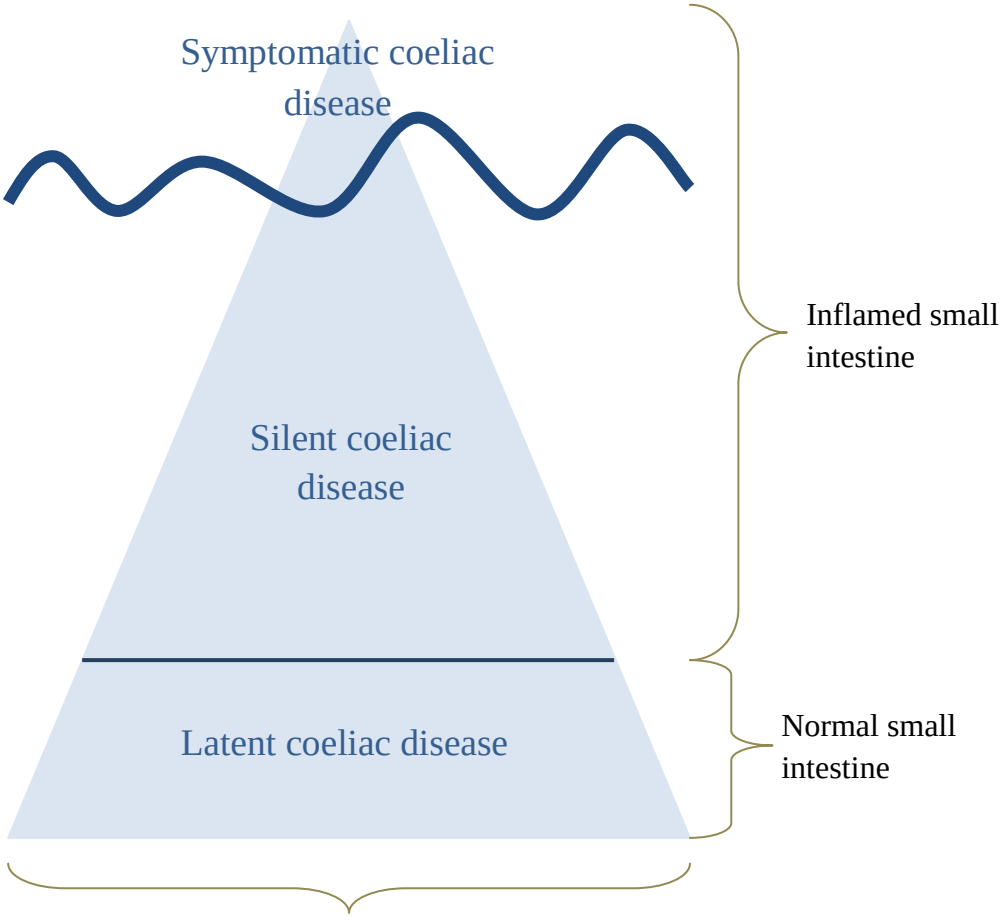
Approximately 50% of coeliac patients do not have clinically significant diarrhoea and present with less severe symptoms such as lassitude, weakness or weight loss. Anaemia is a frequent finding in coeliac disease and may be the presenting feature (Halfdanarson 2007). Microcytic anaemia, secondary to iron deficiency anaemia is highly prevalent and is frequently the sole manifestation of the disease; oral iron supplementation will prove useless in these cases. Macrocytic anaemia may occur as a result of folate or B_{12} deficiency. This deficiency was previously thought to be unusual in CD because B_{12} is absorbed in the terminal ileum, the distal part of the small intestine, and coeliac pathology primarily occurs proximally. Studies have reported low serum vitamin B_{12} levels in untreated patients (Dahele 2001). Low calcium and vitamin D absorption may give rise to osteomalacia and bone pain, with an increasing risk of developing osteoporosis.

1.3.2 Silent and latent coeliac disease

The true prevalence of CD within a population is difficult to estimate as many affected individuals will not display any symptoms; the disease is therefore undetected. These patients are said to have a "silent" form of the disease- these individuals have positive coeliac serology with an abnormal small intestinal mucosa. "Latent" coeliac disease is a poorly understood condition where individuals are found to have positive serology but normal small bowel biopsy whilst on a gluten-containing diet. Latent coeliac patients do not display any symptoms but they may have had some symptoms in the past or may develop them in later life.

These cases of CD, often undetected, make up a large part of what is now frequently described as the "coeliac iceberg", with clinically diagnosed cases forming only the tip (Catassi 1994) see Figure 1.1 The coeliac iceberg. This concept was emphasised by a multicentre, screening study undertaken in Italy, where it was found that for every diagnosed coeliac patient seven went undiagnosed. Those were subsequently confirmed by biopsy to have the disease (Catassi 1996). Screening relatives of individuals with coeliac disease has increased the number of "silent" cases being diagnosed and treated (Mustalahti 2010).

Figure 1.1 The Coeliac Iceberg



1.4 Pathogenesis of coeliac disease

The pathophysiology of coeliac disease is not fully understood, but what is known is summarised in Figure 1.2 and below.

1.4.1 T-cells in lamina propria

Coeliac disease occurs in genetically susceptible individuals in possession of particular human leucocyte antigen (HLA) class II molecules with approximately 95% of patients expressing human leucocyte antigen (HLA) -DQ2 and the remainder HLA-DQ8 (Sollid 1993). The HLA-DQ2 or DQ8 molecule is responsible for antigen recognition (Sollid 2002). Digested gluten peptides enter the lamina propria of the small intestine either through the enterocytes or paracellularly (Zimmer 2010), where they must bind to HLA molecules on dendritic antigen-presenting cells enabling the peptides to be presented to T-cells. The HLA molecules have a peptide binding groove in which there are discrete binding pockets, found in positions 1, 4, 6, 7 and 9, which can accommodate amino acid side chains. HLA-DQ2 and DQ8 have a preference for binding peptides with negatively charged anchor residues; HLA-DQ2 at positions 4, 6 or 7 (Johansen 1996, van de Wal 1996) and HLA-DQ8 at positions 1 and 9 (Kwok 1996, Suri 2005). Figure 1.3 is a diagrammatic representation of the binding of an HLA-DQ2 molecule to a γ-gliadin epitope that has undergone selective deamidation (Arentz-Hansen 2002).

Gluten proteins contain 30-35% glutamine and 10-15% prolamine residues. Gluten, as a result, contains very few negatively charged amino acids. These proteins undergo a process of partial selective deamidation where certain glutamine residues are converted to glutamic acid, mediated by tissue transglutaminase-2 (tTG2) (Molberg 1998). The presence of many proline residues, resistant to digestive enzymes, ensure that many immunostimulatory QXP motifs survive digestion in fragments comprising of 20-40 amino acid peptides (Shan 2002), increasing the Immunogenicity of the peptide. This allows peptides large enough for T-cell recognition to be presented.

Gluten-sensitive T helper cells proliferate in response to presentation of the antigen and release interferon-γ (IFN-γ) (Nilsen 1995, Troncone 1998). This expands cytotoxic T-cells in the lamina propria. IFN-γ also stimulates fibroblasts to release matrix metalloproteases (Daum 1999) which degrade the extracellular matrix, and also stimulates disease-specific plasma cells to produce anti-tissue transglutaminase antibodies which are initially released into the lamina propria (Sollid 1997). It is these enzyme antibodies in the blood stream that

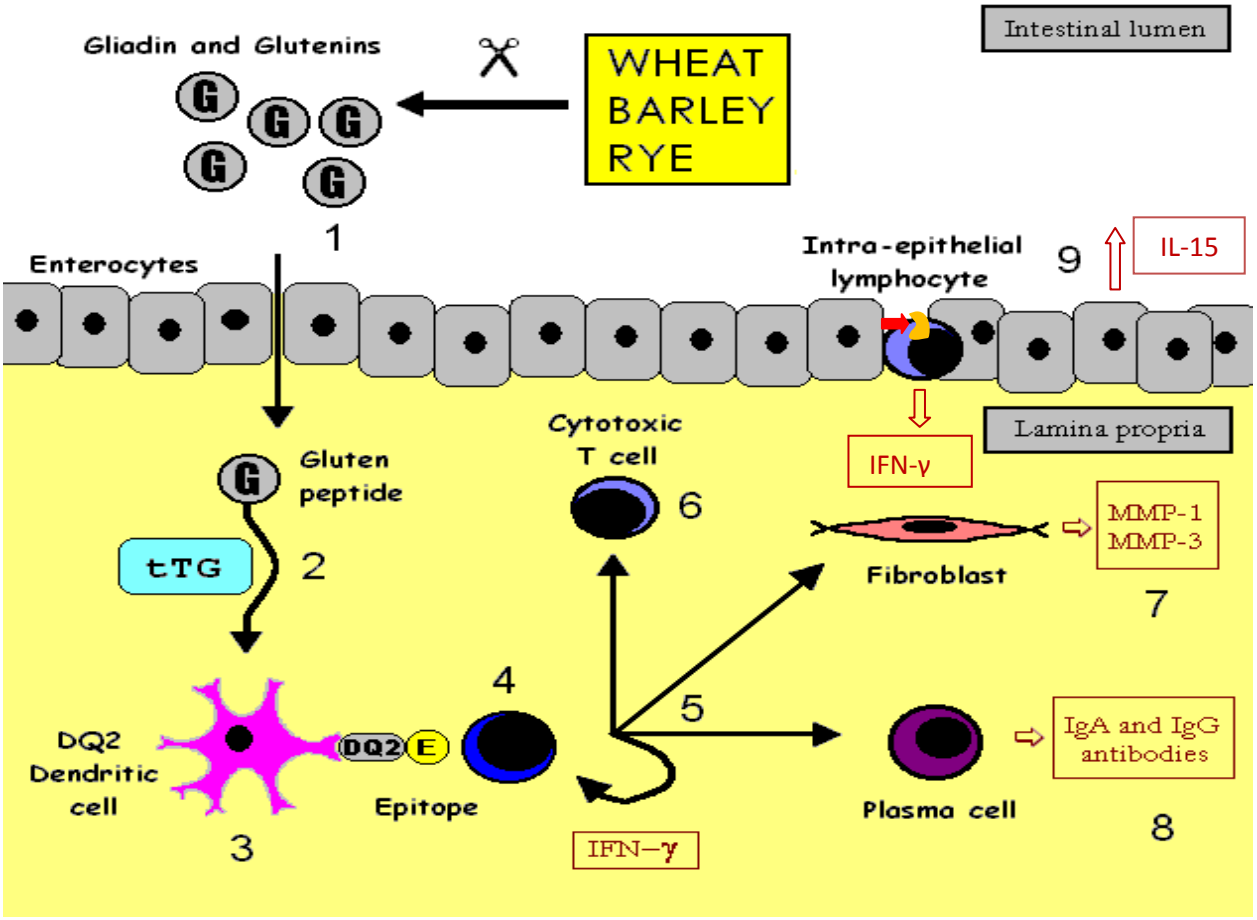
can be quantified to screen for coeliac disease as a prelude to referral for diagnostic endoscopy and distal duodenal biopsy, to permit histological evaluation of the small intestinal mucosa.

1.4.2 Intra-epithelial lymphocytes

Intra-epithelial lymphocytes (IELs) are localised between intestinal epithelial cells and are thought to play an important role in immune surveillance of the epithelium. They are an abundant and heterogeneous population of T cells that are upregulated in coeliac disease. They are specialised CD8 positive lymphocytes which can respond to antigens via a T-cell receptor, or natural killer (NK) cell receptors. The NK cell receptors NKG2D and CD94 are thought to kill enterocytes via MICA and HLA-E ligands expressed on the surface of enterocytes under stress, whereas NKG2A is thought to be protective through activation of TGF-β (Braud 1998). The role of IELs in the pathogenesis of coeliac disease is not fully understood however, they may represent the starting process of coeliac disease (Mäki 2003).

In the normal small intestine, IELs are normally comprised of approximately 75% $CD8^+$T-cell receptor (TCR) $\alpha\beta^+$ and 15% TCR $\gamma\delta^+$ cells (Jarry 1990). There is an expansion of both subsets in active coeliac disease (Kutlu 1993), possibly as a result of raised interleukin-15 expressed and secreted by stressed enterocytes (Maiuri 2000 and 2003, Mention 2003, Hüe 2004, Malamut 2010). These IELs are enriched with cytolytic proteins (Ciccocioppo 2001, Di Sabatino 2001, Oberhuber 1996). They produce large amounts of IFN-γ (Olaussen 2002) that are thought to contribute to the villous atrophy found in untreated coeliac disease. Following initiation of a gluten-free diet, there is recovery of the villous architecture with the numbers of $CD8^+TCR\alpha\beta^+$ IELs returning to normal. In contrast the numbers of $TCR\gamma\delta^+$ IELs remain high for many years afterwards (Kutlu 1993, Bhagat 2008). Current opinion suggests that $CD8^+TCR\alpha\beta^+$ IELs exert a deleterious effect on epithelial cells that can be

Figure 1.2: Summary of key events in the pathogenesis of coeliac disease



1. Gluten peptides, derived from luminal digestion, enter the lamina propria

2. Selective deamidation of specific glutamine residues by tTG

3. Dendritic cells present peptides bound to DQ2

4 Recognition of peptide and proliferation by gluten-sensitiveT helper cells

5. Cytokine production recruits and stimulates effector cells

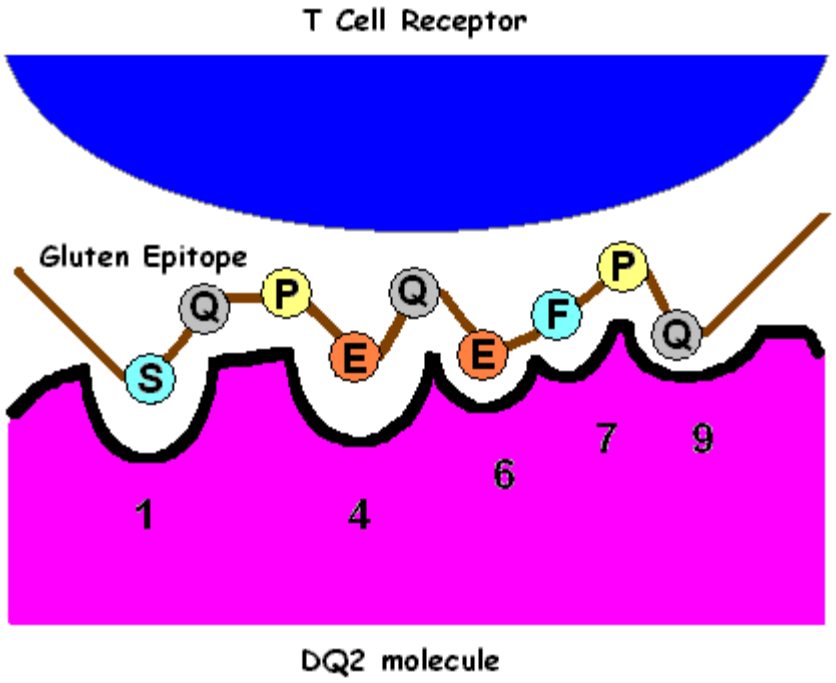
6. $CD8^+$ cytotoxic T-cell population expands in lamina propria and epithelium

7. Fibroblasts secrete matrix metalloproteinases which degrade the extracellular matrix

8. Disease-specific antibody production by plasma cells

9. Intra-epithelial lymphocytes activated with IL-15 and NKG2D expression

Figure 1.3 Diagrammatic representation of the interaction between the DQ2 molecule peptide binding groove and an epitope from γ-gliadin.



Key anchor points are at positions 1, 4, 6, 7 and 9 with negative charges preferred at positions 4, 6 and 7. **E** is the deamidated glutamine residues to glutamic acid.

antagonized by TCRγδ^{+} IELs and that this regulatory function may be overwhelmed in active coeliac disease (Bhagat 2008). Indeed their persistence in coeliac patients on a GFD and the lack of elevation in patients with other small bowel enteropathies suggest a specific relationship between the pathogenesis of coeliac disease and TCRγδ^{+} IELs (Spencer 1989).

The interaction between IELs' natural killer cell receptor NKG2D with MICA, its ligand expressed on enterocytes under stress ensures that a toxic cascade of intracellular processes terminally damage the enterocyte. It is thought that MICA expression on the enterocytes is induced by gliadin, or its p31-49 peptide (see section 1.11.1) via IL-15 (Hüe 2004).Within these enterocytes, exosomes containing granzyme and perforin are released which ultimately destroy the enterocyte (Hüe 2004).

TCRαβ^{+} CD8+ IELs represent an effector T-cell subset that mediates enterocyte cell destruction ultimately leading to villous atrophy in coeliac disease. The basis for this destruction is the recognition of stress ligands expressed on the surface of enterocytes. Whether this killing is mediated mainly by the natural killer receptors or whether activating

natural killer receptors lowers the activation threshold of the TCR allowing IELs to recognise the low-affinity self –antigens expressed by enterocytes remains unanswered. The role of TCR$\gamma\delta^+$ IELs in the pathogenesis of coeliac disease is also unclear. There are no studies at present to conclude definitively that they play no decisive role, have a protective role or contribute to the pathogenesis of coeliac disease.

In order for IELs to kill epithelial cells they need changes in the epithelium resulting in upregulation of IL-15 and non classical MHC class I molecules that are recognised by natural killer receptors as well as activation and expansion of HLA DQ2 or DQ8 restricted gluten-sensitive CD4+ T-cells in the lamina propria producing IL-21 and IFN-γ with other as yet undetermined factors.

The relationship between normal IELs in coeliac disease and those found in refractory coeliac disease is very poorly understood. IELs in type 1 refractory coeliac disease (RCD) express TCR on their surface whereas in type 2 RCD IELs lack surface TCRs, see section 1.7.1. The reason for this as well as the cause is not known and perhaps may represent another disease process leading to a more aggressive disease course. What is interesting is that the genome-wide association searches (see section 1.8) have not found epithelial specific genes associated with coeliac disease, unlike other inflammory disorders such as asthma (Zhang 2012).

1.4.3 Interleukin-15

Interleukin-15 (IL-15) is emerging as an important cytokine in regulating the IEL response in coeliac disease and refractory coeliac disease (RCD), see section 1.7.1. It is able to stimulate two natural killer cell receptors, CD94 and NKG2D, on IELs which are upregulated in active CD (Jabri 2000, Hue 2004, Meresse 2004) as well as MICA on the surface of enterocytes (Hüe 2004). IL-15 is up-regulated by enterocytes and lamina propria monocytes in active CD and RCD, see section 1.7.5. IL-15 drives the differentiation, functional maturation and survival of natural killer $CD8^+$ T-cells, in the lamina propria, and $CD8^+TCR\alpha\beta^+$ IELs (Mention 2003, Di Sabatino 2006). In active CD and RCD enterocyte-derived IL-15 may activate IELs by two possible mechanisms: as a soluble cytokine (Di Sabatino 2006) or more likely, as a membrane-bound cytokine on the surface of enterocytes (Mention 2003, Bergamashi 2008).

Interleukin-15 serves several functions in the epithelium of CD patients. Due to its potent anti-apoptotic effect observed at low concentrations (10 times less than those required for

proliferation), IL-15 stimulates the survival of IELs in uncomplicated CD even more efficiently than that of clonal pre-malignant IELs in RCD. This provides a rationale for their massive accumulation despite a modest proliferation rate (Mention 2003, Di Sabatino 2006). It is also emerging as a driver for epithelial damage. *Ex-vivo*, IL-15 stimulates various effector functions from IELs from either active CD or RCD individuals. Notably, IL-15 stimulates IEL secretion of IFN-γ and their granzyme/perforin-dependant cytotoxicity against enterocyte lines (Mention 2003, Di Sabatino 2006). In addition, neutralising anti-IL-15 antibody inhibits the production of IFN-γ and epithelial apoptosis in organ culture from active CD (Mauiri 2000, Benahmed 2007, Fina 2007), attesting to the important contribution of IL-15 to mucosal damage. Furthermore, it may promote the activation of gliadin-specific IELs in mice (Oh 2004). IL-15 has also been shown to stimulate the spontaneous killing of enterocyte lines by IELs from active CD as well as RCD, where the T-cell receptor is lacking. This suggests that additional receptors may also be involved in the killing of enterocytes (Mention 2003, Di Sabatino 2006). Recent work also indicates that IL-15 may function in this regard by orchestrating the interactions between NK receptors on IELs and their ligands on epithelial cells without involving the T-cell receptor (Meresse 2009).

1.4.4 Dendritic cells

Little is known about the role of dendritic cells in coeliac disease. Dendritic cells are messenger cells with a rapid turnover. They help regulate the intestinal immune respose to the many foreign antigens they come across. Murine CD103+ dendritic cells have been shown to induce gut-homing receptors on responding T cells and their differentiation to FoxP3+ regulatory cells *in vitro*. Induction of regulatory T cells requires TGF-β and convertion of retinal to retinoic acid by CD103+ dendritic cells. A recent study in mice, which had been engineered to express IL-15 in their intestinal mucosa, demonstrated an inflammatory immune response rather than tolerance towards gluten after co-administration of retinotic acid acting via dendritic cells (De Paolo 2011).

Another more recent study suggests that the phenotypic role of dendritic cells can be altered by the concentration of tissue transglutaminase 2 (TTG2) (Dalleywater 2012). At lower concentrations the dendritic cells were able to sample gluten antigens whereas at higher TTG2 concentrations they were able to present antigen to T-cells more effectively.

1.4.5 Potential ideas in the pathogenesis of coeliac disease

This would suggest that there are two mechanisms involved in the pathogenesis of coeliac that may be interlinked. What is not clear is the link between the epithelial and IEL response and the adaptive anti-gluten response. There is an adaptive T-cell response predominantly in the lamina propria as well as the innate response potentially driven by IL-15 and IEL enterocyte interaction. It is perhaps this response that is the initiator of the pathological process with the adaptive T-cell response maintaining the inflammation. Pathological changes in the mucosa of coeliac individuals start to occur one hour after ingestion of gluten (Ciclitira 1984, Maiuri 1996) which is too early for an activated T-cell response. Tissue transglutaminase is an intracellular enzyme that requires active secretion into the lamina propria to deamidate gluten peptides. The more peptides are deamidated the greater the available antigens for presentation to T-cells. It may be that the innate response, dominated by IELs and enterocytes, initiates the inflammatory cycle that is maintained by the adaptive T-cell response. Further understanding of the pathogenesis will allow potential treatments to be designed.

1.5 Diagnosis of coeliac disease

1.5.1 Immunological markers and screening

Serum IgA and IgG antibodies against gliadin, reticulin, endomysium (components of connective tissue), tissue transglutaminase and deamidated gliadin peptides are detectable in individuals with CD. Screening for serological markers is initially used for individuals who are at increased risk of CD: patients with non-specific symptoms; those with a family history of CD or patients with an associated condition such as Type 1 diabetes mellitus.

Serum anti-endomysial antibody (EMA) is measured by incubating sections of monkey oesophagus or human umbilical cord with serum from an untreated coeliac patient and is standardised in Europe. Anti-endomysial antibodies present in the serum will react with the connective tissue antigens which can be detected by a fluoresceinated secondary antibody. IgA EMAs are reported to be the most reliable serological test for CD with a sensitivity of approximately 90% and a specificity of 99% (Stern 2000). False positive results are unlikely, however, the results can be subjective as they depend on the interpretation of the observer as to whether or not the staining is positive or not. The expertise in this technique

is being lost due to time constraints in modern immunology laboratories. The slightly lower sensitivity of EMA probably reflects the high prevalence of selective IgA deficiency among coeliac patients. Approximately 2-3% of coeliac patients are IgA deficient (Cataldo 1998) which can produce false negative EMA results. For this reason, it is recommended that serum IgA levels be measured in those individuals at a higher risk of CD, those with symptoms or with a family history. IgA deficient individuals should be screened using an IgG-EMA.

Prior to use of EMAs, measurements of serum anti-gliadin antibodies (AGA) were used to detect CD. However the test for AGA is less sensitive and specific and can give a positive result in non-coeliac individuals with conditions such as gastroenteritis, inflammatory bowel disease and cow's milk protein intolerance (Uibo 1993).

In 1997, Dieterich and colleagues identified tissue transglutaminase (tTG) as being the autoantigen against which anti-endomysial antibodies are directed. Consequently, an IgA anti-tTG ELISA was developed which utilised a purified extract of guinea pig liver. This assay was reported to be cheap, easy to perform, less time consuming and less subject to inter-observer variation than the IgA EMA (Dieterich 1998). The diagnostic efficiency of the antibody screening tests currently available was compared in a multi-centre study (Stern 2000). The anti-tTG IgA ELISA was found to be less specific than the IgA EMA test (95% compared to 99%) but more sensitive (93% compared to 90%). An anti-tTG assay that employs human recombinant tTG as the coating antigen is more specific than the guinea pig liver ELISA (Tonutti 2003, Lewis 2006).

Antibodies to deamidated gliadin peptides (DGP) have been shown to be of diagnostic value (Volta 2010). However, a meta-analysis comparing the performance of DGP test with the tTG test, the current standard, suggested that the tTG test outperforms the DGP test (Lewis 2010a). Where this test may be useful is in assessing gluten intake on a gluten-free diet. Monzani *et al* (2011) demonstrated both IgA and IgG anti DGP assays showed higher sensitivity than anti-tTG IgA and AGA IgA in monitoring compliance with GFD in children with coeliac disease, but anti DGP IgA paired with IgG seemed to perform better. They did not outperform anti-tTG IgA for CD screening.

A combination of IgA tTG and IgG DGP may increase sensitivity for screening coeliac patients as it picks up IgA deficient patients.

1.5.2 Histology

The coeliac lesion predominantly affects the proximal small intestine with lessening damage occurring towards the distal small intestine. The pathology spans a spectrum of severity that has been classified into five stages by Marsh (1992): the pre-infiltrative, infiltrative, hyperplastic, destructive and hypoplastic (atrophic) lesions. These were subsequently modified by Oberhuber (1999) to be able to use the classification for diagnostic purposes. The classification is summarised in table 1.1. The parameters assessed in the diagnosis of coeliac disease on mucosal small intestinal biopsy specimens include villous or crypt architectural changes as well as lamina propria cell density and intra-epithelial lymphocyte (IEL) cell counts.

Table 1.1 The modified Marsh classification

	Type 0	**Type 1**	**Type 2**	**Type 3a**	**Type 3b**	**Type 3c**
IEL/100 enterocytes	<40	>40	>40	>40	>40	>40
Crypts	normal	normal	hypertrophic	hypertrophic	hypertrophic	hypertrophic
Villi	normal	normal	normal	mild atrophy	marked atrophy	absent

These features form a continuum with normal mucosal architecture at one end and the classical flat lesion at the other end, which may take many years to develop. A comparison of normal duodenal mucosal biopsy with Marsh 3c duodenal mucosal biopsy is seen in Figure 1.2. Marsh 0 is a normal small intestinal biopsy. Marsh 1 is an increase in IELs counted per 100 enterocytes, the first and most sensitive index of the effects of gluten on the mucosa. Infiltration of gluten-dependent lymphocytes into the lamina propria is also seen. However, it is not pathognomonic for coeliac disease as they may be raised in infections for example. Marsh 1 is often seen in dermatitis herpetiformis (see section 1.7.8), in treated coeliac disease, and in family members of those affected by coeliac disease.

Marsh 2 is an infiltrative lesion where there is an increase in crypt length as well as raised IELs. The crypt epithelia are infiltrated by lymphocytes. It is not often seen in the diagnosis of coeliac disease.

Marsh 3 is the destructive lesion Marsh described. It is divided into three different subgroups depending on the degree of villous atrophy. These lesions are the classical

"diagnostic" lesions of coeliac disease, before the advent of serology and newer genetic screening. Mitotic activity within the crypt is substantially increased and the time taken for cells to migrate from the crypt to the surface is reduced from between three and five days to between one and two days. Consequently, surface epithelial cells are immature and the surface enterocyte height is reduced. All type 3 lesions have raised IELs and an increase in crypt depth. Marsh 3a is characterised by mild villous flattening; Marsh 3b is characterised by marked villous flattening, and Marsh 3c is characterised by a flat mucosa. All are evidence of an advanced cell-mediated mucosal immune response which occurs in untreated coeliac disease, whether silent or classical. However, villous atrophy is not pathognomonic for coeliac disease as it can occur in the context of other disorders such as cow's milk allergy, giardiasis, rotavirus infection and autoimmune enteropathy (Freeman 2004).

Type 4 is a very rare hypoplastic lesion which is characterised by a flat mucosa but normal crypt depth and normal IEL count. It is probably a historic lesion seen in severely emaciated small children and most likely signifies malnutrition parallel findings in kwashiorkor (Brunser 1970).

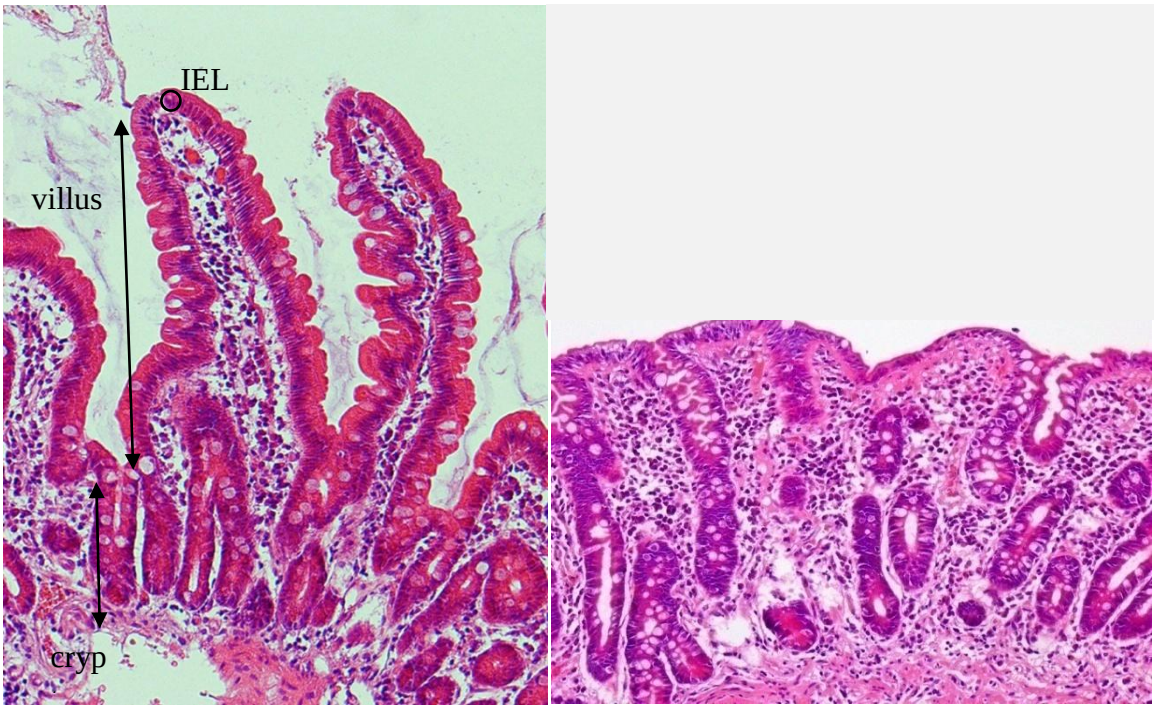
1.5.3 ESPGHAN criteria for diagnosis of Coeliac Disease

The required criteria for a diagnosis of CD were first proposed by the European Society of Paediatric Gastroenterology and Nutrition (ESPGHAN) in 1970 (Meeuwisse 1970). The original statement proposed that a diagnosis should be based on three criteria:

i. Structurally abnormal jejunal mucosa when taking a gluten-containing diet
ii. Clear improvement of villous structure when taking a gluten-free diet
iii. Deterioration of the mucosa as a result of a gluten challenge.

Figure 1.4 Histological changes of coeliac disease

Marsh 0 **Marsh 3c**



H+E stain second part of duodenum low power (x100) courtesy of Dr Chang, St Thomas Hospital

A patient underwent a minimum of three endoscopies for a definite diagnosis of CD. The criteria were revised in 1990 (Walker-Smith 1990) after it was found that many gastroenterologists were not recommending a gluten challenge and a third biopsy. The revised criteria continue to emphasise that an initial biopsy is paramount. Histological examination showing the characteristic abnormalities, that is, lymphocytic infiltration, villous atrophy and crypt hyperplasia, on a gluten-containing diet provides the first step towards a diagnosis. In children under the age of 2 a gluten re-challenge and small intestinal biopsy is still required for the diagnosis of coeliac disease.

A definite symptomatic improvement within a few weeks of commencing a strict gluten-free diet would be consistent with a diagnosis of CD. A second biopsy may be carried out 3 to 6 months after commencement of the diet to confirm that there has been histological improvement as well as symptomatic relief (NICE guidelines 2009 CG86).If there is a symptomatic improvement but no histological recovery the GFD should be verified and continued and a further biopsy taken after three months. It has been shown that complete mucosal recovery can take up to two years (Grefte 1988, Wahab 2002). Where there is still

no histological response or symptomatic improvement it is recommended that patient compliance to a gluten-free diet should be examined.

Those patients who do not respond to a strict diet may have refractory sprue, ulcerative jejunitis or intestinal lymphoma (see sections 1.7.5, 1.7.6, 1.7.7). A third endoscopy after gluten challenge is no longer a compulsory diagnostic step. It may be required however at a later date if the original diagnosis of coeliac disease is brought into question.

However, at present a consultation process is underway to further revise these ESPGHAN criteria in view of more accurate serological tests and knowledge of genetic risk factors. Only 12% of paediatric gastroenterologists, who responded to a postal survey, adhered to the current ESPGHAN guidelines (Ribes-Koninckx 2011). The main transgression was the omission of gluten re-challenge in the under 2 age group. The algorithm proposed by the ESPGHAN working group on the diagnosis of coeliac disease is complicated. It aims to reduce the need for endoscopy which requires anaesthesia in the paediatric cohort. The authors propose that children who have classical symptoms of coeliac disease, with HLA-DQ2 or -DQ8, the genetic predisposition, and strongly positive serology should not undergo endoscopic evaluation of the small intestine. Those who have the genetic predisposition and equivocal serology should proceed to a biopsy of the small intestine, as in those patients in whom the diagnosis in unclear. The new proposed algorithm includes the use of genetic screening, a test that is currently not widely used in routine practice (Husby 2011).

1.6 Treatment

1.6.1 Gluten-Free Diet

Once a diagnosis of CD has been established, patients are advised to start a lifelong gluten-free diet (GFD). This involves the exclusion from the diet of any cereal that contains wheat gluten and prolamins from rye and barley or its derivatives, such as malt, or hybrid wheat varieties, such as spelt. The place of exclusion of oats from a gluten-free diet remains controversial, see section 1.6.1.1. A typical gluten-containing diet contains an estimated 10-20 grams of gluten, derived from multiple sources and therefore a GFD necessitates a calculated avoidance of many foods. A strict gluten-free diet is low in dietary fibre and

folate, niacin and vitamin B_{12} (Thompson 2005, Thompson 2000, Thompson 1999). Complying with a gluten-free diet is difficult for a number of reasons:

1. Gluten may contaminate food during harvesting, food preparation or processing (Ellis 2008b, Hernando 2008)

2. Gluten-free products are more expensive than gluten containing products and may be more difficult to source (Singh 2011)

3. Dietary compliance is poor particularly in adolescents (Fabiani 2000)

4. There is no agreed consensus on the minimal amount of gluten permitted in food: in Australia "no detectable gluten" is <5mg/kg (Australia and New Zealand Food Standards Code 2003) whereas in Europe "gluten–free" is <20mg/kg (Codex Stan 118 1979 amended 2008)

5. A wide variety of gluten sensitivities exist between patients: 50% of patients experience symptoms with an acute challenge whilst 50% do not

6. To improve palatability, many gluten-free products contain purified wheat starch which invariably contains residual gluten (Ellis 2008b)

7. A gluten-free diet does not resolve the problems associated with the rare complication of refractory coeliac disease, see section 1.7.5

Patients not adhering to a gluten-free diet are predisposed to numerous sequelae such as short stature (Bonamico 1992), nutritional deficiencies, early osteoporosis, secondary autoimmune disorders (West 2004, Lewis 2010b), malignancies (Holmes 1989), infertility and poorer outcome of pregnancy (Freeman 2010). However, a gluten-free diet has few side effects.

Naturally gluten–free cereals include rice, millet, maize and sorghum (reviewed in Ellis 2002). Quinoa, an Andean grain from Peru has many benefits for a gluten-free diet as it is the only plant to have all essential amino acids as well as being high in minerals and vitamins however, quinoa has similar prolamin content to wheat (Vega-Gálvez 2010). A recent study demonstrated that not all cultivars of quinoa are safe for coeliacs to eat as evidenced by T-cell activation (Zevalos 2012).

Products labelled as gluten-free have to adhere to strict permissible levels of gluten in Europe (Codex Stan 118 1979). This standard was amended in 2008 to reduce permissible

level of gluten in gluten-free foods. The change was ratified in January 2009, however, manufacturers had until January 2012 to comply. Foods labelled as "gluten-free" must now contain no more than 20mg/kg of gluten whereas foods labelled as "very low gluten" can contain between 21 to 100mg/kg.

1.6.1.1 Oats in gluten-free diet

Oats are more distantly related to wheat (more details in section 1.9.1) and are thought to be less toxic to coeliac patients. Several studies suggest that oats are safe for coeliac patients to eat (Janatuinen 1995, Janatuinen 2002, Koskinen 2009), however, there are high drop out rates in both the Janatuinen studies in the oats group of patients. Janatuinen in his five year follow up study (2002) only had 23 out of the original recruited 35 coeliac patients left, and in his study in 1995 there was a 10% drop out rate in the group eating oats. Many commercially available oat products are contaminated with wheat, rye or barley (Thompson 2004). Studies from Norway suggest that in a small minority of patients oats are toxic (Lundin 2003, Arentz-Hansen 2004). These studies are with oats that are known to be free from gluten contamination that are produced specially for coeliac patients in fields where no harmful cereals are grown. The oats are processed in a separate production line and repeated testing using ELISA kits have shown that the oats are free from contamination. In 19 coeliac patients recruited for Lundin's study, one was found to develop new villous atrophy, the blistering rash of dermatitis herpetiformis, associated with coeliac disease, and clinically active coeliac disease. Subsequently Arentz-Hansen (2004) found two further patients who deteriorated after they started eating oats following changes to the national recommendations. Biopsies from these two patients were taken and challenged with oats resulting in avenin reactive T-cells. These cell lines were also tested with wheat and found to stimulate the T-cells but through different epitopes.

1.6.2 Nutrient replacement

Nutritional deficiencies should recede on commencement of GFD but dietary supplements may be necessary. Recovery from anaemia can take between 6 and 12 months as intestinal mucosa reverts to normal (Annibale 2001). After a diagnosis of CD patients should undergo a dual-emission x-ray absorptiometry (DXA) scan to assess bone mineral density (BMD). Patients found to have a low BMD should be advised to strictly adhere to a GFD, to avoid smoking and excessive alcohol intake, and take calcium and vitamin D supplements (BSG Guidelines 2007).

1.6.3 Novel therapies

A greater understanding of the pathogenesis of coeliac disease has allowed alternative treatments to be designed to act either as an adjuvant to a gluten-free diet, allowing for minor transgressions, or replace it altogether through potential blocking of various postulated mechanisms (reviewed in Donnelly 2011). The options include:

1. Modifying wheat in an attempt to reduce the toxic gluten epitope content
2. Degrading gluten to smaller peptides using enzymes, not able to be presented to gluten-sensitive T-cells, either *in vivo* or *in vitro*
3. Targeting tight junction proteins to ensure that they do not allow gluten to pass through
4. Tissue transglutaminase 2 blockade to reduce the deamidated peptides presented to gluten-sensitive T-cells
5. Reducing HLA-DQ peptide presentation to gluten-sensitive T-cells by amino acid substitution of gluten T-cell stimulatory sequences
6. Immune system modification either by naturally occurring gluten peptide sequences or helminth infection
7. Targeting inflammatory cytokines with biological therapies, such as anti-IL15
8. Vaccination with gluten peptides in order to induce immune tolerance
9. Intra-nasal administration of T-cell immunostimulatory gluten epitopes to induce tolerance

Many of the options above are a long way off from market as they are in the early stages of development and testing in animal or human subjects. However, this is a rapidly expanding market driven, in part, by the wish of coeliac individuals for an alternative to a gluten-free diet. The main drawback facing researchers is that a gluten-free diet has few side effects. Therefore, for a treatment to be recommended in the treatment of coeliac disease, it has to have an acceptable low number of side effects. A greater understanding of the pathogenesis of coeliac disease will help increase the potential solutions to this.

1.7 Complications and associations of Coeliac Disease

There are many complications associated with coeliac disease. The most common is non-responsive coeliac disease where individuals with coeliac disease fail to improve on a gluten-free diet. A recent study (Dewar 2012) demonstrated by far the most common cause was continued gluten ingestion by the individual, whether it was inadvertently or deliberately. Other causes of continued symptoms were of small bowel bacterial overgrowth, microscopic colitis, concomitant inflammatory bowel disease and lactase deficiency, often with more than one reason being found for continued symptoms.

Females with coeliac disease have a higher incidence of unexplained infertility (Collin 1996), and have lower birth weight infants (Norgard 1999, Ludvigsson 2001).

There is also an established link with other autoimmune diseases such as type 1 diabetes mellitus (Schuppan 2000, Neuhausen 2008), sharing similar genetic variants (Smyth 2009).

Neurological symptoms may also be experienced by coeliac individuals, mainly ataxia and peripheral neuropathy (Hadjivassiliou 1996, 2006, 2008a). A recently identified tissue transglutaminase 6 may be the cause of these symptoms (Hadjivassiliou 2008b).

Dermatitis herpetiformis is a gluten-sensitive skin disorder which is associated with skin deposition of anti-tissue transglutaminase 3 antibodies (Zone 2011). Few patients suffer malabsorption but some do have abnormal small intestinal histology. The treatment involves a gluten-free diet as well as dapsone, the dose of which may be decreased after initiation of a GFD.

The risk of both enteropathy associated T-cell lymphoma (EATL) and small intestinal adenocarcinoma are elevated, the latter manyfold. A wealth of collected data exists as the incidence of cancer is one of the leading causes of concerns for coeliac patients as well as their gastroenterologists (Cellier 2000, Askling 2002, Catassi 2002, Green 2003. Howdle 2003, Rampertab 2003, Green 2004, West 2004, Smedby 2005, Mearin 2006). The risk of developing these rare cancers occurs with coeliac disease as opposed to silent or latent forms of the disease (Elfström 2011).

1.7.1 Refractory Coeliac Disease

A rare complication of coeliac disease is refractory coeliac disease (RCD), when clinical symptoms and histological changes persist or recur after a good response to a gluten-free diet and after other causes of villous atrophy having been excluded. Intestinal mucosal

recovery on commencement of a GFD can take up to two-years (Grefte 1988) and it may therefore be necessary to delay a diagnosis of refractory coeliac disease until at least one-year on a GFD.

Refractory coeliac disease can be classified according to the immunophenotype of intra-epithelial lymphocytes. Abnormal clonal lymphocytes with loss of surface markers CD8 and CD3 occur in type 2 RCD whereas in type I RCD the majority of lymphocytes have normal surface markers and T-cell receptors are polyclonal (Cellier 2000). Differentiation between the different types of refractory coeliac disease is important as the reported five-year survival rate varies between 40-58% for type 2 RCD (Mauriño 2006, Al-Toma 2007, Malamut 2009, Rubio-Tapia 2009), and 93% in type 1 RCD (Malamut 2009). The main cause of death in type 2 RCD is progression to an enteropathy associated lymphoma (EATL) with a five-year survival rate of 8-20% (Gale 2000, Al-Toma 2007) as well as progressive malnutrition.

Little is known about the pathophysiology of RCD due to the scarcity of these patients in clinical practice making it difficult to conduct randomised controlled trials and tailor treatment.

1.8 Genetics

Coeliac disease has a strong genetic component. At present the most important genetic association with coeliac disease are the HLA-DQ2 and-DQ8 haplotypes (Sollid 1989, Karell 2003). The vast majority of coeliac individuals have the HLA-DQ2 haplotype with approximately 5% of coeliacs being HLA-DQ8. This difference in haplotypes may affect disease presentation and subsequent course (Biagi 2011). HLA-DQ molecules are responsible for antigen presentation, and therefore, it is not surprising that they have the strongest genetic link to coeliac disease. However, whilst HLA-DQ molecules are necessary for coeliac disease, they are not sufficient to cause it. Approximately 30% of healthy individuals in the Western population express these haplotypes (Sollid 1989) and yet do not develop coeliac disease; therefore, other genetic or environmental factors must be involved.

Genome-wide linkage studies aim to identify broad genomic regions which contain predisposing variants for the disease in question. They rely on comparing large sample sizes of healthy controls with the target disease group trying to find genes that are more frequently associated with the disease group. There have been 5 genome-wide association

studies (GWAS) which have added new genes associated with coeliac disease (van Heel 2007, Hunt 2008, Smyth 2009, Coenen 2009, Garner 2009, Dibois 2010). The main function of these genes concern co-stimulation or co-inhibition of T and B-cells, T-cell development in thymus, cytokine and chemocytokine signalling as well as the detection of viral DNA, although the latter had relatively weak statistical significance. These findings implicated primary alterations in the immune response rather than the intestinal epithelial barrier or digestive enzymes, as was previously considered. It is still not clear how genetic change alters biological function, however, it is allowing research to be targeted to the genes involved and increase knowledge of pathogenesis of coeliac disease. Interestingly none of these genes relate to IELs, see section 1.4.2. The findings of the GWAS studies are summarised in Table 1.2.

1.9 Cereal chemistry

1.9.1 Taxonomy

Cereals are species of grasses (gramineae) which are classified into four subfamilies. See Figure 1.5. Wheat (triticum) is a member of the triticeae tribe, of the pooideae subfamily. Within the triticeae tribe a number of species exists: one of the most widely grown species of wheat is *triticum aestivum*, used in bread making. Oats are slightly removed from wheat, rye and barley, being a member of the avenae tribe. The cereals are largely classified according to the structure of their seed-storage proteins; this may partially explain the observed differences in the disease-activating properties of oats and wheat.

1.9.2 Gluten

The environmental trigger for CD is dietary gluten in genetically susceptible patients. Gluten can be further subdivided into two major protein subclasses according to their solubility in alcohol and aqueous solutions. These subclasses consist of gliadins, soluble in 40-70% ethanol and glutenins which are large, polymeric molecules insoluble in both alcohol and aqueous solutions. Glutenins help trap carbon dioxide in the fermentation process to enable the bread to rise while gliadin helps trap water to give bread its elasticity. Frazer in 1959 digested wheat gluten into six fractions using pepsin and trypsin. Frazer's Fraction III (FFIII) is now widely used in coeliac research as it is soluble in buffered saline

solution and contains both gliadins and glutenins. A summary of the preparation is shown below in Figure 1.6.

Table 1.2 New genes implicated in coeliac disease from the genome-wide association studies

Study	Size of sample	Gene	Action
Van Heel 2007	778 coeliacs 1422 controls	IL2	T-cell activation and proliferation
		IL21	Enhancement of B, T, NK cell proliferation and IFN-γ production
Hunt 2008	1643 coeliacs 3406 controls	CCR3	Recruitment of effector immune cells to site of inflammation
		IL12A	Induction IFN-γ secreting Th1 cells
		IL18RAP	Mature IL18 induces T-cell IFN-γ synthesis, RAP expressed in un-stimulated T and NK cells
		LPP	Unknown. Highly expressed in small intestine
		RGS1	Regulation of chemocytokine receptor in B-cell activation and proliferations. Expressed in IELs in coeliac disease
		SH2B3	Regulates T-cell receptor, growth factor and cytokine receptor-mediated signalling in leukocyte and myeloid cell homeostasis
		TAGAP	Expressed in activated T-cells, unknown role in immune function
Smyth 2008	8064 T1DM 9339 controls 2828 families 2560 coeliacs	CTLA4	Expressed on the surface of helper T-cells, transmits inhibitory signals to T-cells
		CCR5	Loss of expression of chemocytokine receptors
		PTPN2	Regulation of cell growth, differentiation, mitotic cycle and oncogenic transformation
		IL7R	critical role in the V(D)J recombination during lymphocyte development, controls accessibility of TCR gamma locus
		PRKCQ	Controls cellular signalling in NF-κB pathway
		CD226	mediates cellular adhesion to other cells bearing its ligands
		UBASH3A	Negatively regulates T-cell signalling

Genes in grey do not quite reach statistical significance however are implicated in the pathogenesis

Table 1.2 New genes implicated in coeliac disease from the genome-wide association studies continued

Study	Size of sample	Gene	Action
Coenen 2009	1368 rheumatoid arthritics 795 coeliacs 1683 controls	PTPN22	Regulates T-cell signalling
		TNFAIP3	Inhibition of NF-κB activation and TNF-mediated apoptosis
		REL	Important role in survival and proliferation
		PFKFB3/PRKCQ	Controls cellular signalling in NF-κB pathway
Garner 2009	906 coeliacs 3819 controls	ITGA4	Cell adhesion, migration and activation of immune cells
Dubois 2010	4533 coeliacs 10750 controls	BACH2	Promotes oxidative cell death
		CCR4	Regulation of cell trafficking of various types of leukocytes
		CD80	Controls strength and nature of the response to T or B-cell receptor activation by antigens
		CIITA-SOCS1-CLEC16A	Transcriptional co-factor in HLA class II positive cell lines
		ICOSLG	Controls strength and nature of the response to T or B-cell receptor activation by antigens
		ZMIZ1	Regulation of the activity of various transcription factors
		ETS1	Key player in thymic $CD8^+$ lineage differentiation, promotes RUNX3 expression
		RUNX3	Master regulator of $CD8^+$ T lymphocyte development in the thymus
		THEMIS	Positive and negative T-cell selection during late thymic development
		MMEL1/TNFRSF14	Widespread functions in peripheral leucocytes and promoting thymocyte apoptosis

Genes in grey do not quite reach statistical significance however are implicated in the pathogenesis

The gliadins and glutenins can be further subdivided into groups according to their molecular weight as shown in Figure 1.7. The gliadins are well described as toxic (Dicke 1953a,b, van de Kamer 1953, Sturgess 1994, Shidrawi 1995, Anderson 2000, Arentz-Hansen 2000, Ellis 2003, Fraser 2003, Martucci 2003, Mazzarella 2003). However, the glutenins have only recently been extracted in a pure form (Keck 1995). Prior to this the glutenins were assumed to be non-toxic. Any observed toxicity was thought to be due to gliadin contamination with no evidence to support this theory (van de Kamer 1953).

Figure 1.5: Cereal taxonomy of the grass family (Gramineae)

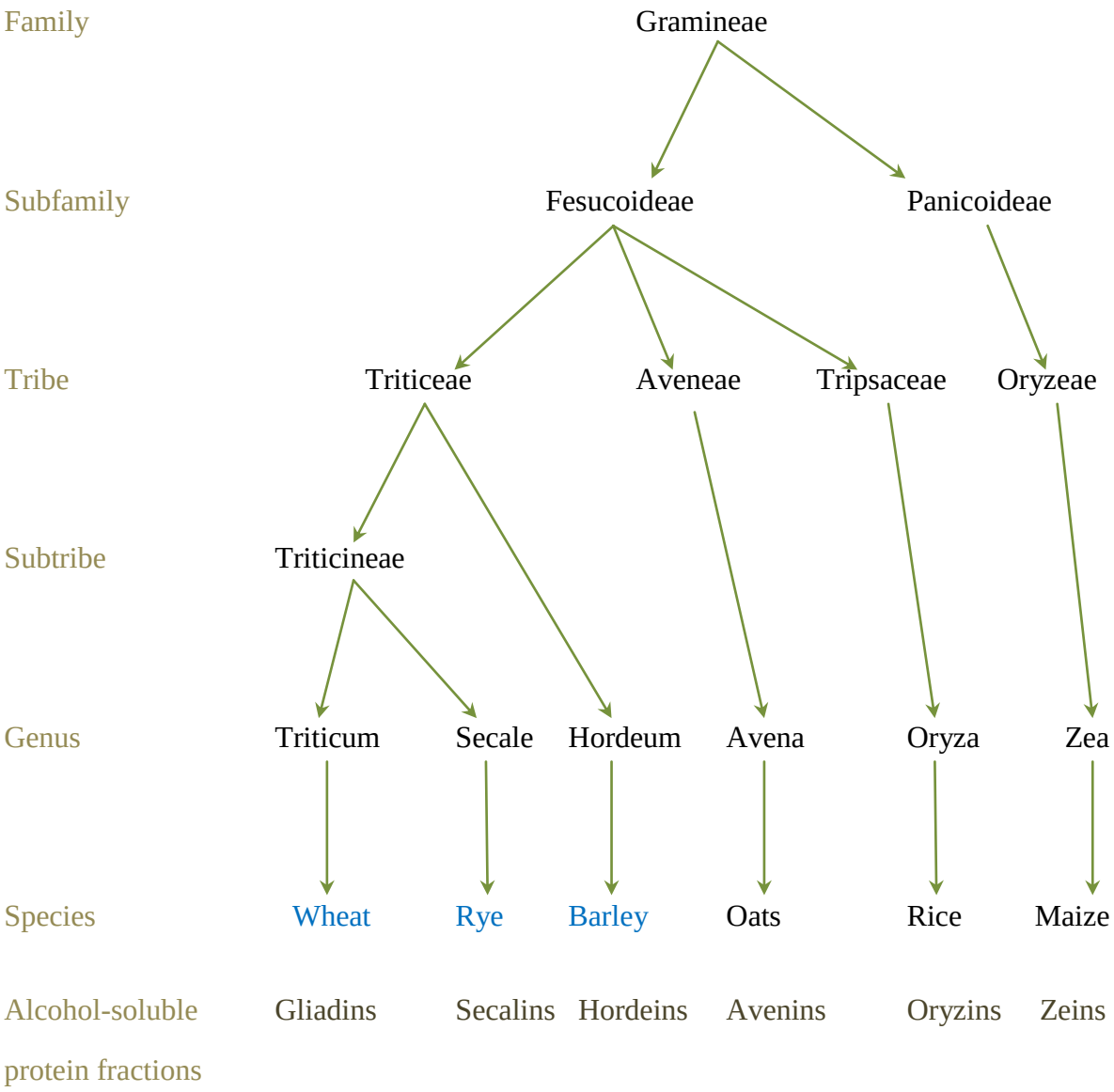


Figure 1.6 Frazer's enzymatic fractionation of gluten

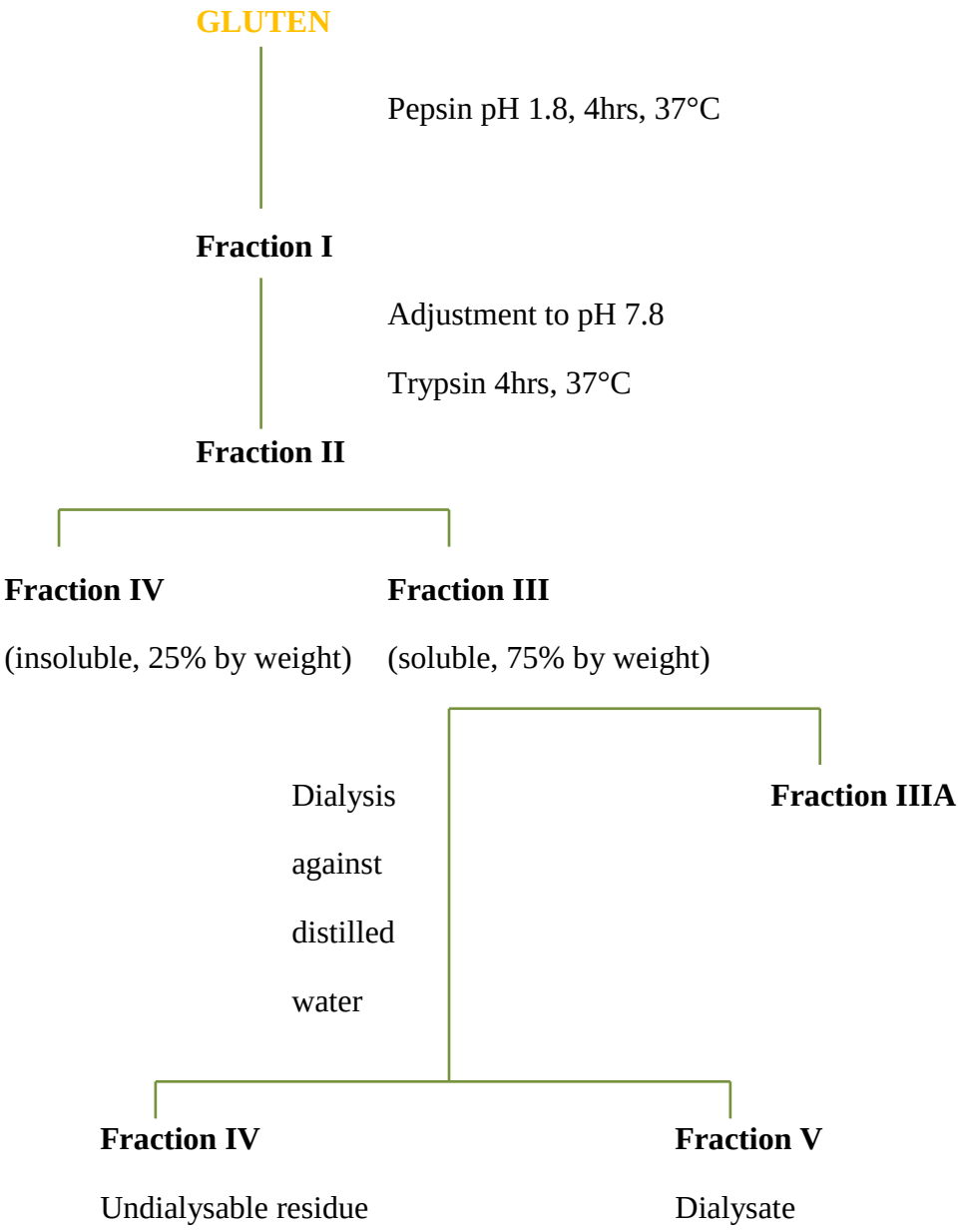
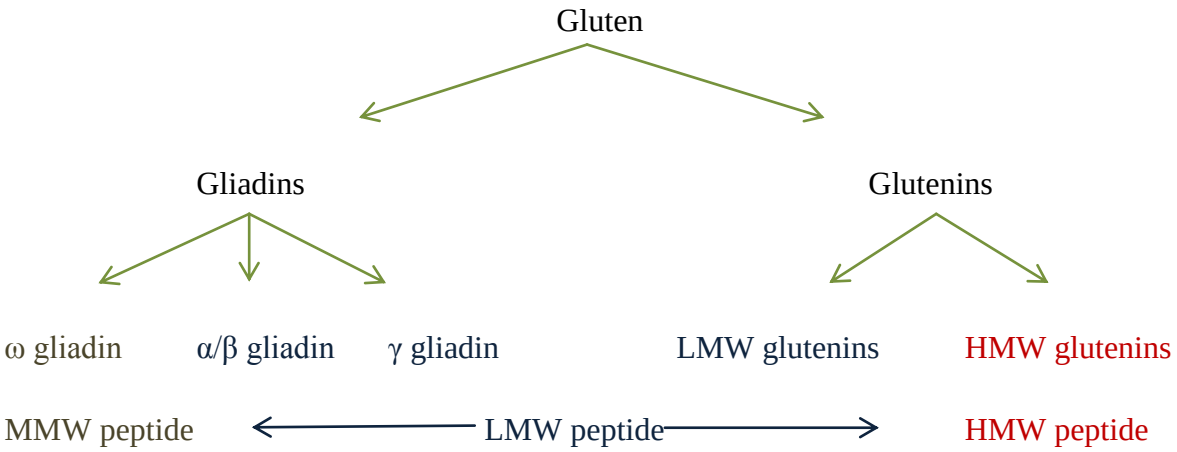


Figure 1.7 Wheat protein classification



We now know that the glutenins are toxic (van de Wal 1999, Vader 2002a, Molberg 2003, Dewar 2006, Ellis 2006). Vader tested small intestinal T-cell clones of children with coeliac disease with an array of proteins and peptides, including low molecular weight (LMW) glutenin. Four out of 16 T-cell clones from children were sensitive to the LMW glutenin. Van de Wal tested different 18 amino acid peptide sequences spanning the entire HMW glutenin molecule with a T-cell clone from an HLA-DQ8 positive paediatric coeliac patient and was able to demonstrate the minimal epitope required for optimal T-cell stimulation was residues 724-734, QGYYPTSPQQS. However the drawback in both these studies is that the reaction with T-cell clones is too specific and unlikely to represent the mass action of T-cells present in the gut. Dewar demonstrated the toxicity of a mixture of chemically purified HMW glutenins by demonstrating *in vitro* stimulation of T-cell lines in 11 of 17 coeliac patients. He also performed *in vivo* challenges in 3 patients with coeliac disease taking small intestinal biopsies every hour for six consecutive hours after instillation of the HMW glutenin into the duodenum. All patients developed significant change in intestinal morphology after 4hrs. We still know little about the toxicity of the HMW subunits known as Dx5, Bx7, By9 and Dy10. Molberg's group (2003) managed to stimulate 5 out of 12 human leucocyte antigen (HLA)-DQ2 T-cell lines when testing recombinant Dy10 and 3 out of 12 HLA-DQ2 T-cell lines with recombinant Dx5. Our group demonstrated the toxicity of recombinant Dx5 and Dy10 fractions by *in vitro* and *in vivo* testing (Ellis 2006). Four out of 13 small intestinal T-cell lines were stimulated by Dx5 and 3 out of 11 T-cell lines were stimulated by Dy10. Both coeliac patients in the *in vivo* studies were found to react however, only one patient was tested with each fraction.

Tye-Din (2010) has recently extended the evidence for immunostimulatory T-cell epitopes in wheat, rye and barley. In his study he tested a large number of potential T-cell epitopes against activated peripheral blood lymphocytes in HLA-DQ2 positive coeliac individuals following a five-day oral gluten challenge prior to venesection. The T-cell response to these epitopes was measured by interferon-γ ELISpot test. He found that the T-cell response differed between coeliac individuals, as well as depending on the gluten challenge cereal, wheat, rye or barley. The strongest immune response, as evidenced by IFN-γ secretion, was from the 33-mer α2-gliadin p56-88. However, the universal response, the most widespread T-cells activation, was from ω-gliadin. Tye-Din's work suggested that the glutenins were not major T-cell epitopes in HLA-DQ2 restricted patients. However, when T-cells did respond they secreted large amounts of IFN-γ.

Classically gluten referred to the storage proteins in wheat gliadins. Rye and barley have similar toxic peptide sequences to gliadin, although less toxicity work has been done as it is assumed that they would also be toxic (Vader 2003). Vader used T-cell lines and clones from 8 different patients with coeliac disease to test 11 amino acid peptides from a selection of epitopes from rye and barley similar but not identical to the equivalent region in gliadin. He found that seven of eleven peptides were recognised by gluten-specific T-cells lines or clones from coeliac patients. No further work has been done to extend or improve these results to date. Now the term gluten is commonly used to refer to the immunostimulatory proteins in wheat, rye and barley

1.9.3 Role of Cereals in bread making

It is the unique protein composition of wheat that makes it suitable for baking leavened bread. It is thought that the prolamins become rearranged during the kneading process, and strong non-covalent bonds form the gluten network. The gluten network and starch granules then serve to trap the carbon dioxide produced by yeast fermentation during bread making (Shewry 1992). The suitability of a wheat flour for bread making is largely determined by its composite glutenin proteins. The structure of the glutenin polymers, the types of glutenin subunits present and the ratio of high: low molecular weight subunits are the major factors contributing to the overall quality of the glutenin protein. The correct balance of viscosity and elasticity is essential and it is the glutenins that are responsible for the elastic properties of the dough (reviewed in Anjum 2007). Rye and barley do not have the equivalent gluten protein content as wheat and are therefore not suitable for bread

making. Barley is mainly used for malt in the production of beer and whisky. Rye is used for making crispbreads, rye breads and is also used in whisky.

1.10 Methods for assessing the toxicity of gluten fractions and peptides

There are animal models available for coeliac disease, although these have inherent problems. Irish setter dogs have a gluten-sensitive diarrhoeal illness with similar histological lesions (Batt 1984). However, the lack of anti-gliadin and anti-tTG2 antibodies and major histocompatability complex (MHC) class II linkage with disease precludes their use as a model for coeliac disease. Captive rhesus macaque monkeys' chow contains gluten with a minority exhibiting chronic diarrhoea, stomach distension and a blistering rash, similar clinical symptoms to coeliac disease. Juvenile macaques seem prone to elevated anti-gliadin antibodies with similar histological lesions in their duodenums, as seen in humans with coeliac disease, which improve on a gluten-free diet (Bethune 2008). However, a Mamu class II association with simian gluten-sensitive enteropathy, while under investigation (Mazumdar 2010), is yet to be found, as is an immune response directed towards the tissue transglutaminase 2 in this disease.

The current toxicity testing in coeliac disease therefore uses human tissue. The testing can be further subdivided into *in vitro* methods and *in vivo* methods.

1.10.1 *In vitro*

1.10.1.1 Organ culture of small intestinal biopsy

Browning and Trier first described a method where small intestinal biopsies were cultured successfully using an *in vitro* organ culture system (Browning 1969). This was subsequently extended to investigate changes in small intestinal biopsies from patients with untreated coeliac disease (Trier 1970, Falchuk 1974) and the parameters used to assess the biopsies were refined to use mean enterocyte cell height (Howdle 1984). Figure 1.8 explains these terms. This method has now been used by a number of other researchers in assessing the toxicity of gluten fractions (Howdle 1984, de Ritis 1988, Przemioslo 1995, Shidrawi 1995, Maiuri 1996, Biagi 1999, Beckett 1999, Martucci 2003, Mazzarella 2003). Interestingly, Maiuri (2003) demonstrated that A-gliadin 31-43 was not able to stimulate gluten-sensitive T-cells but did produce a positive result in organ culture implying that coeliac disease is not purely driven by T-cells and that innate immunity may also be involved. A point that will be discussed later in this thesis.

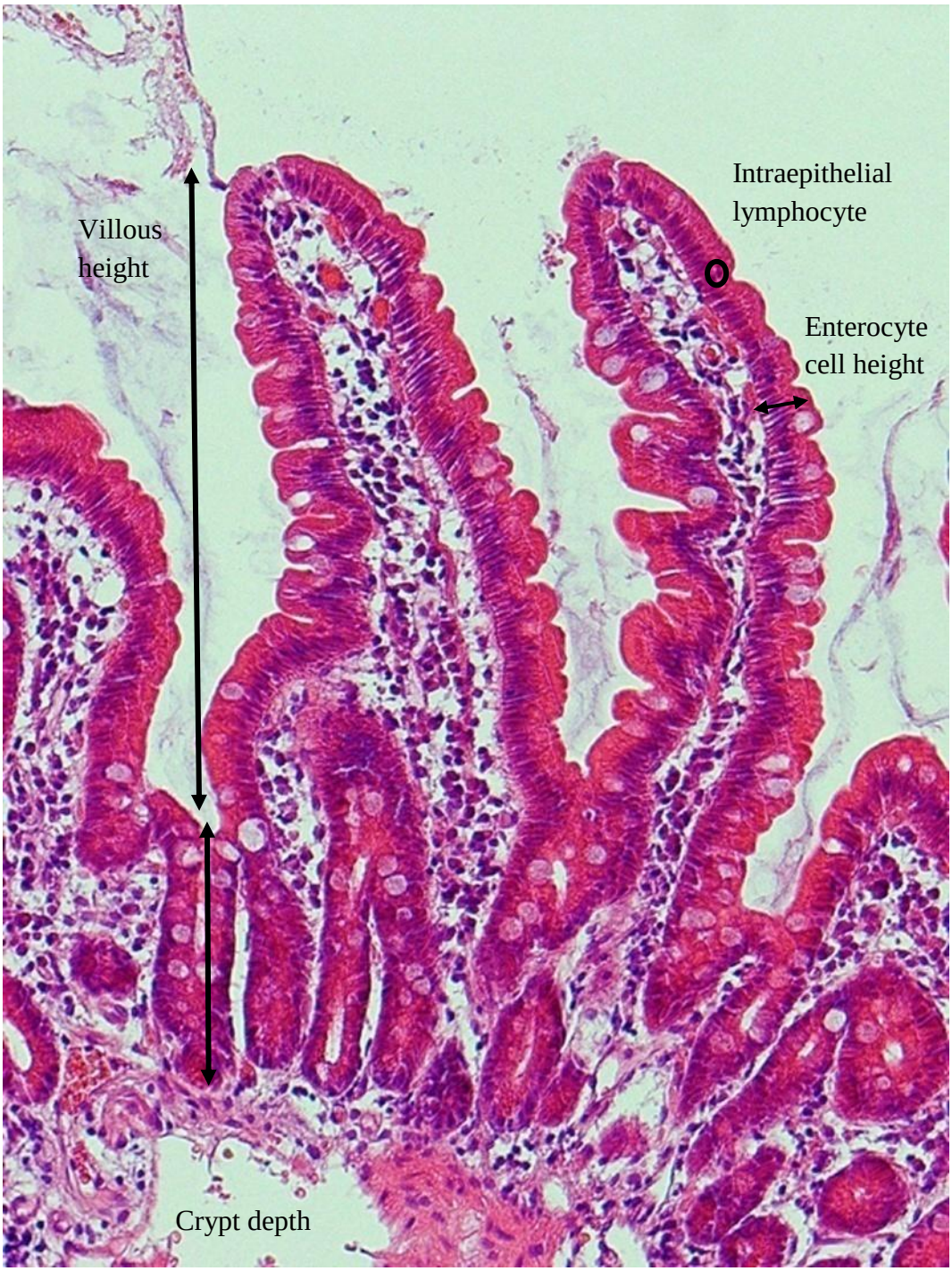
1.10.1.2 Small intestinal T-cell culture

Gliadin specific T-cells were first isolated from coeliac small intestinal biopsies by Lundin in 1993 and grown in culture with cyclical antigenic stimulation. T-cell lines are groups of unselected cells that have only been stimulated a maximum of three times whereas clones are T-cells grown from a single cell. They therefore lack the mass T-cell action of the gut immune system. These cells can then be used for a transformation or proliferation assay.

This assay is used to assess gluten-specific T-cell proliferation in response to the candidate peptide presented by inactivated antigen-presenting cells (APC), usually irradiated peripheral blood mononuclear cells, and compared with a negative control of T-cells with APC. Positive controls of T-cells with APC presenting gluten and T-cells with phytohaemagglutinin-L (PHA) alone are also used. PHA is a plant lectin that triggers lymphocyte agglutination and proliferation.

A stimulation index (SI) can then be calculated after overnight incubation with tritiated thymidine (^{3}H-thymidine) by dividing the mean count per minute of the T-cells with APC and antigen by the mean counts per minute of the T-cells with APC alone. A SI greater than 2 is positive and demonstrates toxicity of the protein or peptide tested. This method has been used to identify the toxic epitopes in gliadin by many researchers (Anderson 2000, Arentz-Hansen 2000, Ellis 2003, Dewar 2006) and continues to be the most widely used form of toxicity testing in this area of research.

Figure 1.8 Parameters used to assess histological changes of coeliac disease mucosal sections



H+E stain duodenum low power (x100) courtesy of Dr Chang, St Thomas Hospital

1.10.2 *In vivo*

1.10.2.1 Small intestinal *in vivo* challenge of gluten

The gold standard for toxicity testing in coeliac disease is *in vivo* gluten challenge as this is the most reliable and relevant method for testing gluten fractions and candidate peptides. Treated coeliac volunteers are sedated and a hydraulic biopsy tube, with a cannula taped to the side, is placed in the second part of the duodenum under x-ray guidance. An exact amount of candidate peptide can then be infused directly into the small intestine and sequential biopsies can be taken. This method has been used to confirm the toxicity of α-, β-, γ- and ω-gliadin fractions (Ciclitira 1984) as well as A-gliadin 31-49 (Sturgess 1994), α-gliadin 57-68 (Fraser 2003) and HMW-glutenin (Dewar 2006). It is an invasive method of testing which requires an experienced physician and relies on the goodwill of the patient. However, it allows a correlation with the T-cell data. This is not widely used as the capsule, hydraulic pump and spare parts required are no longer manufactured.

1.10.2.2 Other *in vivo*

In the search for less invasive *in vivo* methods for testing toxicity of gliadin peptides, other methods have been devised. The rectal mucosa of coeliac patients has been shown to have some of the pathological features of the small intestinal mucosa therefore rectal infusion poses an alternative method which has been used by some researchers (Loft 1990, Ensari 1993). However Loft reported non specific increases in intraepithelial lymphocytes in a few control patients, and patients tend to find it unacceptable (Ellis 2000).

Oral gluten challenge by injection of peptides into the buccal mucosa is relatively non invasive and some people find it more acceptable (Ellis 2000). It has been used by a group in Finland to confirm the toxicity of A-gliadin 31-49 (Lähteenoja 2000a, b).

1.11 Disease activating epitopes identified to date

The immunostimulatory potential of α-, γ- and ω-type gliadins was confirmed using *in vivo* challenge studies (Ciclitira 1984). Since then a great deal of research has gone into identifying the precise regions of gliadin which may be responsible for triggering the disease. One of the earliest studies that attempted to characterise toxic gluten epitopes in CD was of de Ritis *et al* (1988). Using chemical and enzymatic methods to digest A-gliadin, several peptide fragments were produced. By studying the morphological recovery

of paediatric biopsy specimens after 48 hours of culture in medium with and without A-gliadin, peptides corresponding to amino acids 1-127, 128-246, 1-55,1-30 and 31-55 were found to be toxic *in vitro*.

A great deal of attention has focussed on a region of A-gliadin corresponding to amino acids 31-49. Recognition of this peptide by an HLA-DQ2 restricted peripheral blood T-cell clone provided evidence of its potential immunogenicity (Gjertsen 1994). Further studies using small intestinal organ culture showed that this peptide was immunostimulatory to the coeliac small intestine *in vitro* (Shidrawi 1995). A similar peptide (A-gliadin, amino acids 31-43) induced anti-endomysial antibody production from treated coeliac biopsy specimens but not controls (Picarelli 1999). Biagi (1999) abolished the *in vitro* immunostimulatory effects of A-gliadin 31-49 by changing a single amino acid. Perhaps the most significant evidence for a role of A-gliadin 31-49 in disease pathogenesis arose from the *in vivo* challenge study of Sturgess *et al* (1994). Infusion of 200mg of A-gliadin peptide into the duodenum of treated coeliac patients caused a significant decrease in villous height: crypt depth ratio and in the enterocyte cell height, with a parallel increase in the intraepithelial cell counts in all 4 patients tested. A control peptide, corresponding to A-gliadin 3-21, had no histological effects. Subsequent analysis of the biopsies from the Sturgess study used *in-situ* hybridisation to show that exposure to 31-49 caused an increase in the expression of IFN-γ mRNA (Kontakou 1995). This provided robust evidence that the observed histological effects were as a result of activated gliadin-specific T-cells.

Other studies investigating the immunostimulatory potential of this peptide reported conflicting results. The binding affinity of peptide 31-49 for HLA-DQ2 is only moderate (Johansen 1996, Shidrawi 1998) and only one T-cell clone from peripheral blood has been identified (Gjertsen 1994). Arentz-Hansen *et al* (2000) found that polyclonal, small intestinal T-cell lines from 6 patients did not respond to a series of native or deamidated peptides corresponding to A-gliadin 1-58. However, Maiuri *et al* (2003) demonstrated that A-gliadin 31-43 was able to induce damage to small intestinal biopsies in organ culture, mediated by interleukin-15, while failing to stimulate gliadin sensitive T-cells. This suggested that a more complex pathologic situation where the innate immune response was also implicated along with gluten-sensitive T-cells in the histological deterioration of the small intestine in response to gluten peptides.

One of the most significant findings to date was the identification of two HLA-DQ2 restricted T-cell stimulatory α-gliadin peptides corresponding to amino acids 62-75 of α2-

gliadin and 57-68 of α9-gliadin (Arentz-Hansen 2000). Twelve out of seventeen polyclonal T-cell lines had proliferative responses to peptide 57-68, eleven responded to peptide 62-75. In each case deamidation was found to enhance T-cell proliferation. Peptides not containing the α2 and α9 epitopes failed to stimulate gliadin-specific T-cell lines. It was proposed that the T-cell response in HLA-DQ2 restricted patients is primarily focused on these two immunodominant gliadin epitopes. This theory was supported by similar findings by Anderson *et al* (2000) who investigated peripheral blood T-cell responses to a panel of pooled A-gliadin peptides following a five-day oral gluten challenge. A peptide corresponding to amino acids 57-73 elicited T-cell responses in eleven out of twelve patients but not in controls.

The significance of these overlapping T-cell epitopes in disease pathogenesis was highlighted by Shan *et al* (2002). They identified a physiologically stable 33-mer peptide, corresponding to amino acids 57-89 of α2-gliadin, which included the epitopes identified previously by Arentz-Hansen *et al* (2000) and Anderson *et al* (2000). Not only was this peptide highly immunogenic, but it was also found to be resistant to digestion by gastric, pancreatic and small intestinal brush border enzymes. This could suggest that a build up of the peptide occurs in the lumen of the small intestine, providing enough time for tTG deamidation and thereby increasing recognition of the peptide by specific T-cells. Vader *et al* (2002a) identified an additional 6 T-cell stimulatory peptides which suggested that the T-cell response in CD was not directed against a small number of gliadin epitopes. T-cell lines isolated from paediatric coeliac patients each responded to between one and four gluten peptides, representing sequences from both the gliadin and glutenin fractions. They also found that some children responded to gluten peptides in their native, non-deamidated form. The wide range of gluten peptides recognised by T-cells in young patients lead the authors to suggest that epitope focussing may occur as the disease develops. They postulated that in the early stages of the disease, T-cells recognise a wide range of immunogenic epitopes. As mucosal damage occurs, tTG is released causing deamidation of peptides thereby enhancing the T-cell response and the strength of the HLA-DQ2 binding. The gluten-specific response progressively focuses on those peptides with the strongest binding affinity for HLA-DQ2 and T-cell stimulatory capacity.

Sjöström *et al* (1998) identified a T-cell stimulatory peptide corresponding to amino acids 134-153 of γ-gliadin confirming the disease activating properties of the γ-gliadins. An additional three γ-gliadin epitopes and an α-gliadin epitope was identified by Arentz-

Hansen *et al* (2002) using recombinant proteins. During the course of these experiments the authors observed that there were a high proportion of glutamine and proline residues within the stimulatory epitopes. By mapping the numbers of these residues along the length of the α- and γ-gliadin proteins, a clear correlation was observed between proline content and regions containing stimulatory epitopes. The authors speculated the possible reasons for this. The majority of mammalian peptidases are unable to cleave peptide bonds either side of a proline residue. The more proline residues within a protein or peptide, the less it will be subjected to enzymatic digestion. Furthermore, the spacing between proline and glutamine residues has been shown to influence sequences targeted by tTG (Vader 2002b). Sequences preferable for tTG deamidation such as QXP, where X is any amino acid, were frequently found in regions containing T-cell epitopes.

Tye-Din et al (2010) increased the evidence for potential candidate epitopes in wheat, rye and barley in HLA-DQ2 restricted individuals. They used peripheral blood lymphocytes taken after a five-day oral gluten challenge using the relevant cereal to be tested for IFN-γ enzyme-linked immunosorbent spot (ELISPOT) testing. They tested oligopeptides, 20 amino acids in length, 37 in wheat, including 4 HMW glutenin oligopeptides and one LMW glutenin oligopeptide, 30 in barley and 29 in rye. They demonstrated that the T-cell response *in vitro* depends on the cereal used in the gluten challenge. A hierarchy of peptides was created and those with high IFN-γ secretion were tested with T-cell clones from the small intestine. They showed that there were two responses: the most active peptides as well as the peptides with the widest response in the individuals tested. They further demonstrated that the 33-mer HLA-DQ2 immunodominant peptide (Shan 2002) triggered the highest secretion of IFN-γ, however, ω-gliadin peptides triggered a lower immune response in a wider selection of coeliac patients. They confirmed previous conclusions that the immune response of each coeliac individual is heterogeneous (Vader 2002a) but were able to demonstrate that T-cell clones from individuals challenged with any gluten cereal reacted to two epitopes of ω-gliadin.

Studies investigating the HLA-DQ8 restricted gliadin epitopes are few as only a small proportion of coeliac individuals carry this tissue type. Van de Wal *et al* have identified two HLA-DQ8 restricted T-cell stimulatory epitopes within the gliadins and glutenins (1998, 1999). More recently the same group investigated the effects of the identified HLA-DQ8 restricted gliadin epitope on small intestinal organ culture specimens from HLA-DQ8^{+} individuals. Immune activation and FAS expression in response to this peptide was

detected in six out of six HLA-DQ8^{+} individuals but not in HLA-DQ2^{+} individuals (Mazzarella 2003). The authors concluded that the peptide was probably an immunodominant HLA-DQ8 epitope. However the peptide was only tested in six patients and was not tested with any gliadin specific T-cell lines or clones. Table 1.3 summarises the immunostimulatory epitopes to date.

Table 1.3 Immunostimulatory evidence for epitopes

Name	**Stimulating Amino Acid sequence (epitope)**	**Reference**
α2 gliadin 62-75 α9 gliadin 57-68 HLA-DQ2 immunodominant peptides	PQPQLPYPQPQLPY QLQPFPQPQLPY	Anderson 2000 Arentz- Hansen 2000 Vader 2002a Ellis 2003 Fraser 2003
A-Gliadin 31-43 Innate response peptide	LGQQQPFPPQQPY	Shridawi 1994 (p31-49) Sturgess 1994 Kontakou 1995 (p31-49) Picarelli 1999 Maiuri 2003
A-Gliadin 203-220 HLA-DQ8 immunodominant peptide	QYPSGQGSFQPSQQNPQA	Mazzarella 2003
A-gliadin 51-70	SQQPYLQLQPFPQPQLPYSQ	Martucci 2003
α20-gliadin	PQPFRPQQPYPQPQPQ	Vader 2003
γ30-gliadin 222-236	VQGQGIIPQQPAOL	Vader 2002a
γ1-gliadin	PQQPQQSFPQQQRPF	Vader 2003
γ 2-gliadin	QQPFPQQPQQPFPQ	Vader 2003
HMW glutenins	Not known, possibly GQQGYYPTSPQQS	Van de Wal 1999 Vader 2002a Molberg 2003 Dewar 2006
LMW glutenins	Not known, possibly PPFSQQQQSPFSQQQQ	Vader 2002a

1.12 Choice of peptides and immunogens for the present study

The HMW glutenin glut 04 (Q**GQQGYYPTSPQQS**G) peptide 721-735 (HMW glut$_{04}$) was chosen due to evidence using T-cell clone work (van de Wal 1999) as well as T-cell line and monoclonal antibody work (Mitea 2008). It is also contained in the HMW protein subunit Dy10. Van de Wal took the entire HMW glutenin molecule and divided it into overlapping peptides, 18 amino acids long. He then tested these peptides with a gluten-sensitive HLA-DQ8 restricted T-cell clone from a single child. The letters in bold highlight the optimal stimulating epitope for the clone in van de Wal's study. Mitea found that the

minimum stimulating epitope that monoclonal antibodies recognised was QGQQGYYP, which differed slightly to the minimum stimulating epitope that T-cells recognised, QGYYPTSPQ. In this study, the peptide containing Mitea's minimum stimulating epitopes for both B and T-cells which also had a high activation count in van de Wal's research was chosen, QGQQGYYPTSPQQSG. This peptide configuration had the second highest activity in van de Wal's work was used as, according to protein chemists from GenScript, this was the more stable peptide to make. During the course of this thesis Tye-Din (2011) published his work using ELISpot testing of activated peripheral blood mononuclear cells. He tested a variety of HMW peptides and the peptide labelled W24 in his study contained the HMW glutenin peptide used in this thesis.

Work done previously in our laboratory, raising monoclonal antibodies against peptides of HMW glutenin highlighted some problems (Šuligoj 2011). In order overcome these problems, use of multiple antigenic peptides (MAP) as the immunogen had been suggested by the MRC laboratory in Cambridge. These are large molecules with a central, inert core of branched poly-lysine residues to which the peptide of interest is attached, either 4 or 8 times depending on the size of peptide used, (Posnet 1989) which collectively affords molecular mass large enough to trigger an immune response (see section 6.1). However the peptide used in HMW $glut_{04}$ MAP had to be changed as the chemists from Cambridge Peptides making the MAP, considered the original peptide, QGQQGYYPTSPQQSG, would form ring structures in MAP format and would be difficult to make. The peptide QPGQGQQGYYPTSPQ was used as a compromise, having the 5^{th} highest activity in van de Wal's work.

The LMW glutenin peptide (LMW glt_{156}) 44-59 PPFSQQQQSPFSQQQQ was chosen, the glutamines underlined requiring deamidation. The choice was determined by transformation assays using T-cell clones in children (Vader 2002a). In this study, Vader tested a number of different peptides and protein fragments, some were known to be immunostimulatory while others were novel. Vader tested two novel LMW glutenin peptides LMW glt_{156} and LMW glt_{17}. The LMW glutenin peptide chosen for the studies in this thesis had the highest stimulation index and was able to stimulate more childrens' T-cell clones than the other LMW glutenin peptide tested. The numbers were small however, with only 4 out of 16 T-cell clones stimulated for LMW glt_{156} versus 3 out of 16 for the other LMW glutenin fragment glt_{17}. There were no problems with the manufacture of MAP for this peptide.

1.13 Aims

Van de Wal (1999) and Vader (2002a) have identified potential HLA DQ8 coeliac immunostimulatory epitopes in children in both high molecular weight and low molecular weight glutenins by testing against gluten-sensitive T-cell clones. Neither of these epitopes has been tested in T-cell lines or in small intestinal organ culture.

I wish to further assess the immunostimulatory potential of high molecular weight glutenin $glut_{04}$ and low molecular weight $glutenin_{156}$ in coeliac individuals and type 2 refractory coeliac individuals by

I. Transformation assays of gluten-sensitive T-cell lines with candidate epitopes
II. Measurement of interferon-γ secretion in the transformation assays
III. Morphometric assessment of coeliac duodenal biopsies incubated with candidate epitopes
IV. Measurement of interleukin-15 secretion from these biopsies

The null hypothesis of these studies is that both the KHW glut04 and LMW glt156 glutenin peptides do not stimulate gluten-sensitive T-cells isolated from the mucosa of coeliac patients. They will also not damage the biopsy of coeliac and type 2 refractory coeliac patients when placed overnight in organ culture sytem.

I wish to raise monoclonal antibodies against these two peptides with a view to producing improved methods of gluten measurement.

Chapter 2: Methods

2.1 Patients

The patients used in this study were either treated coeliac disease on a gluten-free diet or untreated coeliac disease therefore still on their gluten-containing diet. For the organ culture group, patients with type 2 refractory coeliac disease were also included. Treated patients were diagnosed following the revised ESPGAN criteria (Walker-Smith 1990) and were attending endoscopy as part of their ongoing clinical management. Untreated coeliac patients were undergoing endoscopy for suspected coeliac disease either with symptoms or with positive serology. These patients were only included if the histology confirmed the diagnosis of coeliac disease, otherwise these patients were used as negative controls. The diagnosis of type 2 refractory coeliac disease was confirmed if the patients had no other cause of villous atrophy identified, were on a gluten-free diet with negative coeliac serology, and had clonality of their intra-epithelial T- cell receptor (Cellier 2000).

Ethical approval was obtained for all experiments (LREC 05/Q0702/167) and every patient gave their written, informed consent prior to the procedure.

2.2 Prolamins

2.2.1 Enzymatic digestion of whole gluten

Small intestinal T-cells are normally presented with digested gluten peptides by antigen-presenting cells in the gut (Lundin 1993). In order to replicate this spectrum of gluten peptides, industrial gluten was digested with pepsin and trypsin for use in the restimulation of small intestinal T-cell lines and positive controls in both the T-cell studies and small intestinal organ culture studies. The following modified method of Frazer's original method (Frazer 1959, Bolte 1996) was used at 37°C:

1. 1 gram of industrial gluten was dissolved in 50ml 0.1M hydrochloric acid and the pH adjusted to 2.
2. 4000U pepsin-coated agarose beads from porcine gastric mucosa (P0609, Sigma) was added and incubated for 2 hours stirring with a magnetic stirrer.

3. To remove the pepsin, the mix was centrifuged for 20 minutes at 2000 revolutions per minute (rpm) (500g).
4. The pH of the supernatant was adjusted to 7.8 with 1M sodium hydroxide
5. 25U of trypsin-coated agarose beads (T1763, Sigma) was added and incubated for 2 hours at 37°C stirring with a magnetic stirrer.
6. The mix was centrifuged for 20 minutes at 2000rpm (500g) to remove the trypsin.
7. 1M hydrochloric acid was added to adjust the pH of the supernatant to 7.0.
8. The pepsin trypsin digest was dried in a glass petri dish at 37°C, scraped off and stored at room temperature.

2.2.2 Antigen preparation

Two groups have reported that deamidation of peptides is crucial for T-cell recognition of peptides (Arentz-Hansen 2000, Anderson 2000). This can be achieved *in vitro* by pre-incubating peptides with tissue transglutaminase (tTG). For the T-cell studies all gluten fractions and control proteins were pre-treated with tTG prior to incubation with the antigen-presenting cells (APC) in the transformation assay. This included PT gluten and Frazer's fraction III (FFIII). The method used was as follows:

400µg/ml of antigen and calcium chloride ($CaCl_2$, C3881, Sigma), to a final concentration of 1mM in phosphate-buffered saline (PBS, P4417, Sigma), were incubated with 100 µg/ml guinea pig liver tissue transglutaminase (T3598, Sigma) for 4 hours at 37ºC.

The mix was then irradiated for 30minutes to sterilise it.

For tTG treatment of synthetic peptides the following recipe was used:

500µg/ml peptide and $CaCl_2$ to a final concentration of 1mM in PBS were incubated with 100µg/ml guinea pig tissue transglutaminase for 4 hours at 37ºC.

The mix was irradiated for 30 minutes to sterilise it.

FFIII and PT gluten were not tTG pre-treated for the organ culture experiments as deamidation has been shown to occur by endogenous tTG within the mucosal tissue in culture (Molberg 2001).

2.3 Small intestinal biopsy organ culture

The organ culture system used in my study differs only slightly from the original method described by Browning and Trier in 1970. Organ culture medium, containing either a test glutenin peptide (200μg/ml) or a control protein (1mg/ml), was prepared two hours prior to obtaining the biopsies and drawn up into 2ml syringes. Culture medium preparations were kept at 37ºC until required. Sterile Falcon organ culture dishes (353037, VWR) were prepared in advance: 1ml of sterile distilled water was added to the outer well, a sterile stainless steel wire grid was placed over the central well and the dishes kept at 37 ºC until required.

Duodenal biopsies were taken using an Olympus GIF H260 endoscope and radial jaw large cap with needle biopsy forceps in the second part of the duodenum. Biopsies for routine histology were taken first. Biopsies used for the culture experiments were immediately placed in warmed (37 ºC) medium which was kept warmed in the hands of the experimenter. These were quickly transported back to the laboratory while being kept at 37ºC.

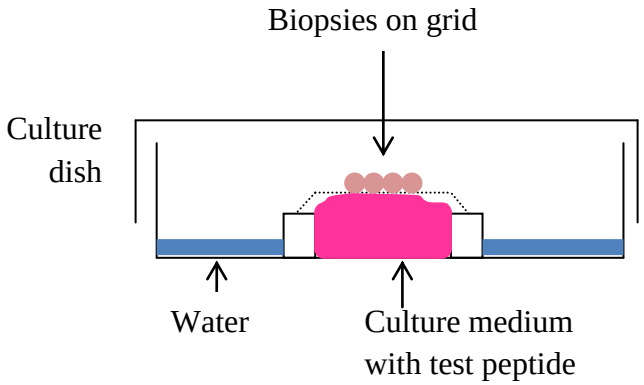
One or two biopsies were carefully arranged on each wire grid so that the villi were uppermost. Harvested duodenal biopsies have a tendency to "curl up" with the villi pointing outwards which helps establish the correct orientation of tissue. The grid was placed over the central well of the organ culture dish and pre-warmed culture medium, with or without the test peptides, was slowly injected into the central well until it was drawn over the tissue by capillary action. Excess medium was drawn back into the syringe so as not to completely submerge the tissue, leaving between 0.5 and 1ml of medium in the central well so that the biopsies are in contact with two thirds of the medium. See figure 2.1 for a cross section representation of this.

The culture dish lid was replaced and the system placed within a sealed chamber. A gas mixture of 95% O_2/ 5% CO_2 was allowed to flow through the chamber for one minute prior to sealing the chamber. The chamber was sealed and gas was further introduced until a pressure of 2psi was reached. The time period between excision of small intestine and placement in the organ culture chamber was approximately 10 minutes. The culture system was incubated for 18-20 hours at 37ºC.

Culture dishes were removed from the sealed chamber the following day. Culture medium was aspirated from the central well, the volume measured and kept at -20°C until required

for cytokine analysis (see section 2.5). The wire grids with the biopsy material were placed onto blotting paper allowing the excess medium to be removed. Biopsies were placed on aluminium foil to be weighed and then fixed in 10% formal saline (see appendix II) for 48 hours. The biopsies were embedded in paraffin wax, sectioned and stained with haematoxylin and eosin (640401 and 640385, Clin-Tech) described in section 2.6, and anti-IL-15 (AF315, R+D Systems), as covered in section 2.5.

Figure 2.1 Cross section of organ culture dish



2.4 T-cell studies

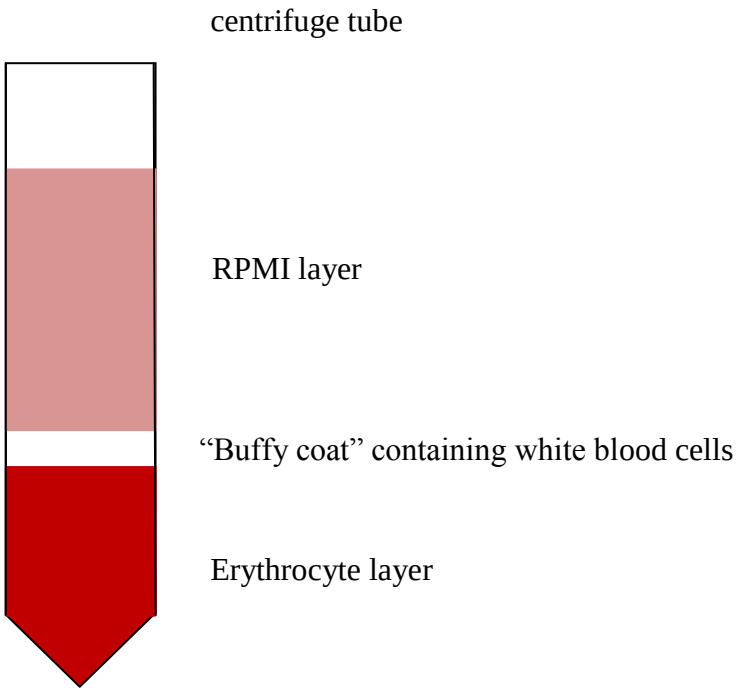
2.4.1 Isolating peripheral blood mononuclear cells from blood

After giving their written, informed consent, 50ml of blood was collected from patients at endoscopy. Blood was taken in a sterile syringe and transferred to a sterile Falcon 50ml tube (352098, SLS) containing 100µl heparin (1000U/ml, LEO Laboratories) and mixed thoroughly. The protocol used for separating the mononuclear cells as follows:

1. Bloods was centrifuged at 3000rpm (1200g) for 10 minutes.
2. The plasma "supernatant" was removed, leaving the white cell interface layer intact.
3. The remaining cell mixture was gently mixed and 10ml was transferred to a 50ml Falcon tube
4. 28ml Rosewell Park Memorial Institute (RPMI) medium (E15-840, PAA) was added and mixed thoroughly
5. The RPMI/blood mix was carefully under-laid with 10ml Lymphoprep (1114545, Axis-Shield PoC)
6. The mix was centrifuged at 3000rpm (1200g) for 20minutes.

7. White cells contained in the grey “buffy coat” under laying the pink RPMI layer were collected using a squeezing pipette and transferred to a 50ml Falcon tube, see figure 2.2.
8. The white cell mix was centrifuged at 2000rpm (500g) for 15 minutes
9. The supernatant was carefully poured off.
10. The resulting cell pellet was loosened and resuspended in autologous plasma medium (ASM).
11. The peripheral blood mononuclear cells (PBMC) were carefully frozen down for future use (see method for freezing/thawing cells, section 2.4.7).

Figure 2.2 Identification of buffy coat after centrifugation 3000rpm (1200g) for 20 minutes



RPMI Roswell Park Memorial medium

2.4.2 Obtaining autologous plasma for culture medium

Plasma was heated at 56 ºC for 35 minutes to inactivate complement proteins and then centrifuged at 3000rpm (1200g) for 10 minutes. The plasma supernatant was collected to use in autologous plasma medium (ASM) or stored at -20 ºC.

2.4.3 Isolating T-cells from *in vitro* prolamin-challenged duodenal biopsies

The establishment of small intestinal T-cell lines *in vitro* was based on methods from Molberg *et al* (2000). Duodenal biopsies were obtained from both untreated and treated coeliac patients using the organ culture method as described earlier in section 2.3. Four to six biopsies were set up on a single stainless steel grid in the organ culture system and cultured overnight at 37 ºC in organ culture medium (see appendix 2 for recipe) containing either FFIII or PT gluten (5mg/ml), as the resulting T-cell line was to be tested for reactivity to glutenin peptides. Following overnight incubation, biopsies were finely minced with two sterile scalpel blades. The minced biopsy material was run through a 70µm filter (352350, SLS) to remove non-degradable material and epithelial cells. This was labelled as the debris fraction and resuspended in autologous serum medium after washing twice in 10ml ASM warmed to 37 ºC and centrifuging at 1500rpm (300g) for 7 minutes. The filtered cells as well as the liquid from the organ culture plate were labelled as the SIL fraction, resuspended in 10ml autologous serum medium and washed twice by centrifuging at 1500rpm (300g) for 7 minutes. The supernatant was discarded and the cell pellet was resuspended in 1ml autologous serum medium with $1x10^6$ irradiated (22 gray) autologous PBMC and 10U/ml human, recombinant interleukin-2 (IL-2, I2644, Sigma) in one well of a 48 well plate (353078, SLS).

2.4.4 Maintenance of T-cell lines in culture

10 U/ml IL-2 was added to the T-cell culture on day one, and at three day intervals subsequently. Medium was refreshed every 2-3 days depending on cell growth rate. This involved carefully removing 1ml of medium and replacing it with fresh ASM and IL-2 at 10U/ml, ensuring that the cell layer at the bottom of the well was not disturbed. On day seven the culture was restimulated as follows: $1x10^6$ autologous, irradiated autologous PBMC were incubated for 18 hours with the stimulating antigen, either PT gluten or FFIII (100µg). Unless otherwise stated it can be assumed that all whole gluten antigens such as PT gluten or FFIII are pre-treated with tTG. T-cells were counted and were resuspended in 1ml of medium containing pre-pulsed PBMC in a ratio of 1:1. The cell line was thereafter maintained on a weekly stimulation cycle by feeding with IL-2 (10U/ml) every 3-4 days after refreshing the medium.

RPMI medium contains a bicarbonate buffer and phenol red indicator, the correct colour for the medium is bright orange indicating a pH of 7.2. A pink colour indicates a raised pH which suggests there is insufficient CO_2; yellow medium is an indicator of acid production as a result of rapid cell growth and suggests the culture needs feeding.

To avoid overcrowding when cells were growing rapidly, cell suspensions were split by transferring either half or a third of the cell suspension to a new well. This was then made up to the volume with fresh ASM and IL-2 was added.

2.4.5 T-cell proliferation assay

For the T-cell proliferation assays, autologous PBMC were used as antigen-presenting cells (APC): either fresh PBMC or thawed from cold storage. The PBMC were counted using a haemocytometer and sufficient cells isolated to allow $5x10^4$ cells per well. Cells were suspended in autologous serum medium at 50µl/well. To prevent APCs proliferating in response to antigen, PBMC were irradiated. A dose of 22 grey was found to be sufficient to prevent cell growth (Molberg 2000). Irradiated cells were added to a 96 well U-bottomed plate (353077, Nunc). Each condition was tested in triplicate, or duplicates if cell numbers were low.

In order to assess the cell viability, a test of T-cells with phytohaemagglutinin (PHA) as well as non-irradiated APCs with PHA were included. Phytohaemagglutinin is a potent stimulator of lymphocyte cell division. A test of T-cells with APC alone was included in each assay as a baseline. A negative protein was not included in the assay as previous work done in the laboratory demonstrated no T-cell response raised against ovalbumin (Ellis 2003).

APC were incubated with antigen at 37ºC; the incubation time depended on the size of the antigen. For small peptides, 4 hours was considered sufficient to ensure the antigen was completely taken up and processed by the APCs. A more complex antigen such as PT gluten or FFIII required 18 hours (Molberg 2000). Each test was performed in triplicate; when there were insufficient cells for triplicate tests, duplicate tests were performed. Whole proteins (FFIII, PT gluten) were used at a final concentration of 100µg/ml. Peptides were used at a final concentration of 10µg/ml (unless otherwise specified).

T-cell lines to be tested for antigen reactivity were counted using a haemocytometer and adequate numbers removed to allow $5x10^4$ cells per well, the total number of Tcells

required for the transformation assays was $1.08x10^6$ to allow for wastage. The cell lines were not used after 3 weeks in culture to minimise the risk of a particular T-cell clone dominating the cell lines. Cells were centrifuged (1500rpm (300g) for 7 mins), resuspended in medium and added to the pre-pulsed APC. The well total volume was made up to 200µl/ well before incubation at 37ºC for 48 hours, although 18 hours was considered sufficient if the colour of the medium was indicative of a rapid proliferation response. Tritiated thymidine (^{3}H; Amersham) was added at 1µCu/well (37,000Bq/well) in 20µl medium and the assay incubated at 37ºC for a further 18 hours. Prior to the addition of ^{3}H, 100µl from each well was carefully aspirated, combined for each test condition and stored at -20°C for interferon-γ analysis by ELISA (see section 2.5.1). Fresh ASM was replaced. Plates were harvested using a Tomtec Cell Harvester (Receptor Technologies Ltd) and ^{3}H-thymidine incorporation into the nuclei of proliferating cells was measured using a Wallac 1450 MicroBeta Plus liquid scintillation counter (PerkinElmer). Stimulation indices (SI) for each antigen were calculated by dividing the mean counts per minute (CPM) for the T-cells plus APC plus test antigen by the CPM for T-cells plus APC alone.

2.4.6 Freezing of cells for liquid nitrogen storage

Cells to be frozen were centrifuged, resuspended in fresh medium and cooled on ice. To prevent rupture of cell membranes, ice cold 20% dimethyl sulphoxide (DMSO, D2650, Sigma), in autologous plasma medium, was added drop-wise to the cold cells until a final concentration of 10% was reached. 1ml aliquots of the cell mixture were frozen in Nunc cryotubes (368632, VWR)to -20ºC overnight and then to -70 ºC the following day for at least two hours before transferring to liquid nitrogen for indefinite frozen storage.

When required, cryotubes were removed from liquid nitrogen and thawed rapidly under running cold tap water. One ml of of ice-cold RPMI medium was added very slowly dropwise with continual shaking, followed by a further 9ml more rapidly with continual shaking to dilute the DMSO. The cell solution was centrifuged at 1500rpm (300g) for 7 minutes and the resulting cell pellet resuspended with 11ml RPMI medium to be further centrifuged at 1500rpm (300g) for 7 minutes. The cell pellet was resuspended in ASM.

2.5 Cytokine analysis

2.5.1 Interferon-γ ELISA

Supernatants from the proliferation assays were analysed for interferon-γ (IFN-γ) production using a non-competitive (sandwich) enzyme-linked immunosorbant assay (ELISA). A hundred microlitres from each well was removed, and replaced with fresh ASM, prior to the addition of tritium in the proliferation assay (see section 2.4.5). The supernatants from each of the test peptides were combined in order to facilitate measurement. A commercial kit (DY285, R+D Systems) was used to measure IFN-γ concentration using the methodology described here.

The IFN-γ ELISA employed human recombinant IFN-γ as a standard and two antibodies: a mouse anti-human IFN-γ capture antibody and a biotinlyated goat anti-human IFN-γ detection antibody. All analyses were carried out on the same day and the lowest sensitivity of the assay was 7pg/ml.The protocol is described in brief below; all steps were carried out at room temperature and all products were supplied in the kit apart from bovine serum albumin (BSA, A7030, Sigma) and phosphate-buffered saline (PBS, P4417, Sigma).

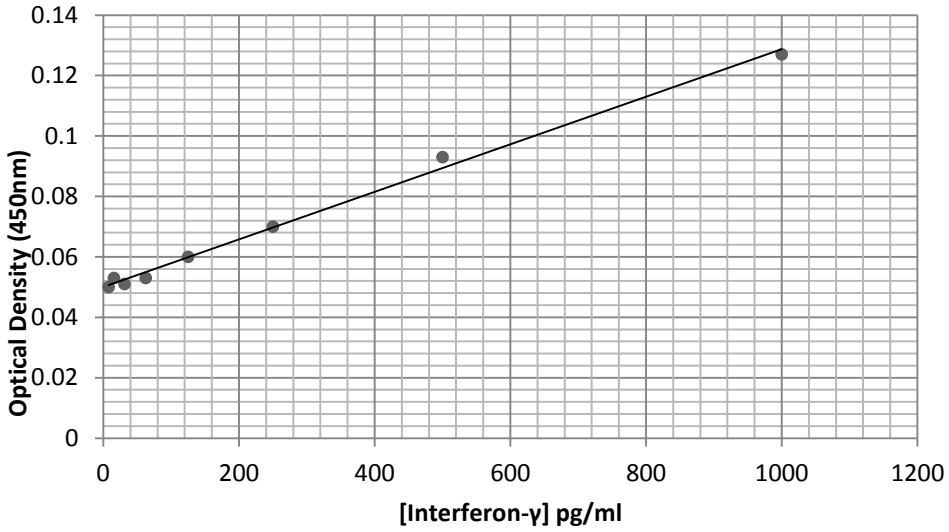
1. A 96 well microtitre plate (430341, SLS) was coated with 100µl capture antibody
2. The plate was sealed and incubated overnight
3. The plate was blocked with 400µl 1% w/v BSA in PBS for an hour
4. 100µl of sample or standard was added and incubated for 2 hours
5. 100µl of detection antibody was added and incubated for 2 hours
6. 100µl of Streptavidin-HRP was added and incubated for 20 minutes in the dark
7. 100µl of substrate solution was added (1:1 mix of hydrogen peroxide and tetramethylbenzidine, DY999 R+D Systems)
8. 50µl of stop solution was added (supplied in the kit)

The plate was washed thrice with PBS/1%v/v Tween 20 (T9039, Sigma) after steps 2-6.

Positive control was supplied in the kit as human recombinant IFN-γ and used to generate a standard curve for each plate. A negative control of ASM only was used. The optical density of each sample was determined using an ELISA reader (Biotec ELx800) with the wavelength set at 450nm. Using the standard curve obtained, the concentration of cytokine in each sample was calculated. Figure 2.1 is an example of the standard curve obtained. The net secretion of interferon-γ was calculated by subtracting the concentration obtained from

a well containing ASM only from the concentration measured in the combined supernatents from each well.

Figure 2.3 A typical standard curve from the low sensitivity IFN-γ ELISA



2.5.2 High sensitivity interleukin-15 ELISA

The level of interleukin-15 (IL-15) secretion from small intestinal organ culture experiments was known to be low therefore an assay with a higher sensitivity was used for these experiments. A commercial kit was used (DY1500, R+D Systems). All the analyses were carried out on the same day with the lowest sensitivity of the kit detecting 3.9pg/ml. The IL-15 high sensitivity ELISA plate already came pre-coated with mouse monoclonal antibody against human IL-15 and employed two antibodies: a standard of recombinant humanised IL-15 and a detection antibody of mouse anti-human IL-15. All reagents, apart from PBS (P4417, Sigma), were supplied in the kit, the protocol in brief, was as follows, all steps were carried out at room temperature:

1. A seven point standard curve was prepared by doubling dilutions from 250pg/ml solution of IL-15 in organ culture medium
2. 100µl per well of either standard or sample was added to the pre-coated microplate and incubated for 3 hours
3. 200µl of Streptavidin-HRP was added to each well and incubated for 45 minutes
4. 200µl of substrate reagent (1:1 mix of hydrogen peroxide and tetramethylbenzidine, DY999 R+D Systems) was added to each well and incubated for 30 minutes

5. 50µl of stop solution (2M sulphuric acid) was added to each well

The plate was washed with wash buffer (PBS/1%v/v Tween 20) four times after steps 2 and 3.

Positive control was supplied in the kit as recombinant humanised IL-15 and used to generate a standard curve for each plate. A negative control of ASM only was used. The optical density of each sample was determined using an ELISA reader (Biotec ELx800) with the wavelength set at 450nm. Using the standard curve obtained, the concentration of cytokine in each sample was calculated as picograms secreted per weight of tissue after the concentration of ASM was subtracted from each value obtained from the standard curve.

2.6 Histological assessment of formalin-fixed, paraffin-embedded coeliac small intestinal tissue sections

2.6.1 Fixing of tissue in formalin

Tissue that has been fixed in 10% v/v formal saline has better preservation of morphology than frozen sections. After overnight incubation with either the control proteins or the candidate peptides (see section 2.3), biopsies were weighed on aluminium foil, placed villi uppermost on 0.45µm cellulose nitrate membrane filter (FDC-860-132G, Whatman Fisher Scientific) and submerged in 20ml of 10% formalin. They were incubated at room temperature for 48 hours before embedding in paraffin wax for sectioning, see section 2.6.2 below.

2.6.2 Embedding of tissue in paraffin wax

Paraffin wax is immiscible with water therefore tissue must be dehydrated before the molten wax is added. The biopsies were wrapped in filter paper and placed in labelled embedding cassettes with stainless steel covers. These cassettes were then placed in graded ethanol baths at room temperature. The small intestinal biopsies were immersed in 400ml 70% v/v 74OP ethanol, followed by 400ml 95% v/v 74OP ethanol and then in 400ml 100% v/v 74OP ethanol each time for a minute. Finally the biopsies were immersed in three different 400ml xylene baths for a minute each. The biopsies on the membrane were then angled at 45° to the bottom of base moulds where molten paraffin at 58°C was added and left to set on a cold plate using Paraffin Embedding Machine (LS-100+ Embedding Center, Bio-Equip) in St Thomas Hospital Histopathology department.

2.6.3 Cutting of sections onto slides

Sections were cut to 4µm on a Leitz microtome and floated in a water bath onto glass slides. Tissue adherence to the slides was enhanced by using SuperFrost Ultra Plus adhesion slides (631-0099, VWR). The sections were then baked overnight onto the slides at 60°C.

2.6.4 De-waxing of tissue sections

Paraffin was removed prior to staining by heating to melt the wax and immersing cut small intestine tissue sections in xylene and graded ethanols. Sections were heated to 60°C for 15-30 minutes on a slide warmer, until the wax melted. The slides were then immersed in three sequential 400ml baths of xylene for a minute each at room temperature. Slides were placed in graded ethanol baths for a minute each, starting at 400ml of 100% v/v 74OP ethanol and then 400ml 95% v/v 74OP ethanol and 400ml 70% v/v 74OP ethanol. Slides were immersed in a 1l basin in running tap water for 5 minutes.

2.6.5 Hematoxylin and eosin staining

Small intestinal tissue sections were stained for morphometric assessment of enterocyte cell height using Mayer's Haemalum (640401, Clin-Tech) and 1% w/v Eosin (640385, Clin-Tech). Slides were de-waxed as per section 2.5.4. Unless otherwise stated all steps were carried out at room temperature. Tap water in London is weakly alkaline and therefore used instead of a weakly alkaline solution in this protocol:

1. Slides were placed in 400 ml Mayer's Haemalum for 5 minutes
2. They were then placed in running tap water for 5 minutes to "blue" the nuclei
3. The slides were than placed in 400ml 1% v/v acid alcohol (see appendix II) for 3 seconds to help differentiate the nuclei. Acid alcohol was made the day of use for best results.
4. They were then placed in running tap water for a further 5 minutes to "blue" the nuclei
5. The sections were examined under a microscope to assess the density of nuclear staining. If over-stained steps 4 and 5 were repeated, if under-stained then steps 1-5 were repeated until satisfactory staining was achieved. This was seen as dark blue nuclei seen in the sections with little cytoplasmic staining.
6. Slides were placed in 400ml 1% w/v Eosin for 2 minutes

7. These were washed in running tap water for 30 seconds
8. The slides were examined for correct nuclear and cytoplasmic staining under a microscope before dehydrating as per section 2.5.2.
9. Sections were covered with a coverslip and DPX, a neutral mounting medium (610301, Clin-Tech) and dried overnight at room temperature.

2.6.6 Immunohistochemistry

An indirect immune-peroxidase method was used to detect interleukin-15 (IL-15) within the formalin-fixed paraffin-embedded sections of small intestine. This method uses the high affinity of avidin for biotin. A primary antibody was applied to the tissue sections which recognise IL-15. A secondary biotintylated antibody binds to this primary antibody following which enzyme-labelled avidin binds to the biotin. The enzyme used in these experiments, horseradish peroxidise, is developed by a chromogen, 3,3' diaminobenzidine tetrachloride (DAB), to give a brown colouration. The strong affinity of avidin for biotin and the mild biotintylation process make the avidin-biotin methods more sensitive than other direct and indirect methods (Hsu *et al* 1981).

Formalin fixation of tissues conceals antigen binding sites by altering the tertiary structure of proteins. Microwaving formalin fixed sections in a citrate buffer rescues binding sites for antibodies previously considered unsuitable for use in routinely processed material (Shi *et al* 1991). The method works by disrupting cross links in the polypeptide backbone induced by formalin. The metal salts used in the buffer further unfold the protein tertiary structure by the dissociation of hydrogen bonds allowing antigens to be recognised by a specific antibody (Norton 1993). A potential problem with the microwaving step is loss of adherence of tissue sections to the glass slides hence the use of SuperFrost Ultra Plus adhesion slides (631-0099, VWR).

2.6.6.1 Microwave processing of tissue sections

Slides were immersed in 400ml pre-warmed (37 ºC) 0.01M tri-sodium citrate solution (pH6.0) on a glass rack in a microwave proof container. They were then microwaved at 700 watts for 10 minutes, in two timed sections of 5 minutes with replenishment of buffer to prevent slides from drying out. The length of time required for microwaving was calculated in preliminary experiments since this varies between tissues. The length of time was also confirmed with immunohistochemistry technicians at St Thomas Histopathology

department who also agreed with the timings. After microwaving, the slides were taken out of the sodium citrate solution and placed directly into warm PBS (37ºC) prior to staining.

2.6.6.2 Avidin-Biotin-Complex staining method of small intestinal sections

All steps were carried out at room temperature and the tissue sections were not allowed to dry out. A commercial kit for anti-goat horseradish peroxidise-DAB cell and tissue staining (CTS008, R+D Systems) and human IL-15 antigen affinity purified polyclonal goat antibody (AF315, R+D Systems) was used according to the manufacturer's instructions and dropper bottles supplied:

Endogenous peroxide activity was blocked to prevent non-specific background staining by covering the sections with 2 drops of 3% hydrogen peroxide for 5 minutes at room temperature. This was then rinsed with 2ml PBS and gently washed with 2ml PBS for 5 minutes in case the tissue sections were loosened by hydrogen peroxide. To prevent non-specific binding of secondary antibody the sections were incubated with 2 drops of goat serum blocking reagent for 15 minutes. Slides were drained and excess serum was carefully wiped off.

The sections were incubated with 2 drops of avidin blocking reagent (CTS008, R+D Systems part 865009) for 15 minutes. They were then rinsed with 2ml PBS, drained and excess buffer carefully wiped off. Two drops of biotin blocking reagent (CTS008, R+D Systems part 865008) were added. The slides were incubated for 15 minutes, then rinsed with 2ml PBS, drained and excess buffer carefully wiped off. The sections were incubated with human IL-15 antigen affinity purified monoclonal antibody at 7µg/ml. This had been pre-determined by titration until cellular staining was preserved with little background staining. The sections were rinsed and then washed three times in 2ml PBS for 15 minutes per wash. Slides were drained and excess buffer carefully wiped off. One to three drops of biotintylated secondary antibody was incubated with the section for 45 minutes. The slides were then rinsed, washed 3 times with 2ml PBS for 15 minutes per wash, drained and excess buffer carefully wiped off. Two drops of high sensitivity streptavidin-horseradish peroxidase were incubated for 30 minutes. The sections were rinsed, washed three times with 2ml PBS for 2 minutes per wash, drained and excess buffer carefully wiped off. Two drops of DAB chromogen solution were added to cover the entire sample and incubated for 7-20 minutes. The colour development was monitored under a light microscope to ensure proper intensity of staining. The slides were rinsed with distilled water and washed in fresh

distilled water for 5 minutes. The slides were mounted with DPX (610301, Clin-Tech) and dried overnight at room temperature.

2.7 Monoclonal antibody production

2.7.1 Animals

An animal project licence number 70/5688 and a personal licence number 70/21925 was obtained by attending modules 1-5 of the Home Office Personal Licensing Course. All animal work was done under the Home Office severity grading of "mild".

A breeding pair of Balb-C mice were obtained from a designated breeding establishment (Harlan) and bred. The standard rodent diet contains gluten and previous work has shown that better antibody responses are obtained in animals that are not consuming gluten (Ellis 1998). A gluten-free diet from birth ensures the mice are antigen naïve and therefore the mother of the litter, when suckling, was on a gluten free diet and the pups were weaned onto a gluten-free diet (dextrose (off) TD.95148, Harlan).

2.7.2 Conjugation of peptides to tuberculin purified protein

The tuberculin purified protein available (PPD, 2T.U/0.1ml, 12-817-01, Statens Serum Institut), which is also used for mantoux testing, contains glycerol and preservatives. As a purified tuberculin protein derivative (PPD) is required for conjugation to smaller peptides the following method was used to clean the PPD, conjugate it to the relevant peptide and precipitate the conjugated peptide in order to use it in immunisation schedule in section 2.7.3.

As PPD is light sensitive the steps were protected from daylight by either wrapping the beaker in foil or performing the experiments in a dark cupboard. The PPD was added to dialysis tubing (molecular weight cutoff 1000 Daltons, D7884, Sigma) and dialysed at room temperature against three 400ml changes of PBS for 45 minutes to remove glycerol. The volume of PPD suspension was measured and four times that volume were added of -70°C acetone. After an hour of incubation at -70°C, this mixture was centrifuged at 3000rpm (1200g) for ten minutes and the pellet was allowed to air dry before being weighed. A molar ratio of 2:1 of PPD:peptide was calculated and was dissolved in PBS to give a final concentration of 2mg/ml of PPD. Fresh, grade 1 gluteraldehyde (G5882, Sigma) was added to this volume to give a final concentration of 0.05% and incubated for 18 hours at room temperature.

After the overnight incubation, ammonium chloride (A4514, Sigma) was added to give a final concentration of 0.1M and stirred for 30 minutes at room temperature. The final volume was measured and four volumes of -70°C acetone was added. This was incubated at -70°C for an hour before centrifugation at 3000rpm (1200g) for ten minutes. The pellet was air dried before diluting to a concentration of 1mg/ml in normal saline.

2.7.3 Immunisation schedule

Mice, once adult, were immunised in groups of three for each immunogen. They were given subcutaneous injections of multiple antigenic peptides (MAPs) 100µl per mouse (3mg/ml in 0.9% saline) emulsified in a 1:1 v/v ratio with complete Freund's adjuvant (CFA, 642851, MP Biomedicals). This was followed a month later by 100µl per mouse MAP (3mg/ml in 0.9% saline) emulsified in a 1:1 ration with incomplete Freund's adjuvant (IFA, 642861, MP Biomedicals) subcutaneously. A tail bleed was performed 10-14 days afterwards to assess the antibody response, see section 2.7.4 and 2.7.5 below. Two to ten weeks after the tail bleed, animals with a good antibody response were given an intravenous injection into the tail of 50µg peptide dissolved in 100µl normal saline. Mice were sacrificed three to five days later and used for fusion experiments to create hybridomas, covered in sections below.

If mice were being immunised with tuberculin-purified protein derivative conjugates of the peptides then prior sensitisation to the tuberculin protein was required. This was achieved by a 100µl subcutaneous injection of tuberculin protein used for BCG vaccine (0.2-0.8 million bacteria made in 1ml Diluted Sauton SSI, both Statens Serum Institut) 2 weeks prior to the immunisation schedule. The animals were immunised following the same protocol as for the MAPs.

2.7.4 Mouse tail bleed

Ten to fourteen days after the incomplete Freund's Ajuvant subcutaneous injection the mice were venesected by a tail bled to assess the antibody response the immunogen. Prior to this, if required, the mice underwent ear notching by the animal house staff to ensure easy identification of individual mice.

Mice in their cages, with ready access to water, were placed in an incubator at 40°C for half an hour to allow vasodilation. Each mouse in turn was placed in a restrainer device with the tail left free. The distal third of the tail was cut at a 45° angle to the skin with a sterile

scalpel. A few drops of blood were collected in 1.5ml Ependorf centrifuge tubes and the tails blotted with soft gauze to ensure haemostasis. The Ependorf tubes were centrifuged at 13000rpm (10,000g) for 5 minutes using a bench-top centrifuge (MSE MicroCentaur). The serum was collected and diluted 1:100 v/v in 0.9% saline for ease of use in ELISA and stored until further analysis at -20°C.

2.7.5 Tail bleed analysis of antibody response to immunogen

Blank ELISA plates (430341, Nunc) were coated overnight at 4°C initially with 100µl Fraser's Fraction III 25µg/ml in carbonate buffer and subsequently 100µl pepsin trypsin digested (PT) industrial gluten 25µg/ml in 60% ethanol as well as 100µl PT recombinant Dy10, a HMW glutenin protein subunit containing HMW glut04, at a concentration of 3.8µg/ml in carbonate buffer, or 100µl MAP LMW glt_{156} at a concentration of 50µg/ml in 60% ethanol. The plates were washed three times with 250µl PBS/0.05% Tween 20 (P9416 Sigma) and blocked with 200µl 1% bovine serum albumin (A7030, Sigma) for an hour at 37°C. The plates were washed three times with 250µl PBS (P4417, Sigma) /0.05% Tween 20 and covered and stored at -20°C if not being used immediately.

Negative control serum was from Balb-C mice at the time of cull, having previously been immunised with HLA DQ8 immunodominant peptide and found not to have mounted a serological response. The resultant optical density from the serum was multiplied by a factor of 2 in order to have a negative optical density result. Positive control for HMW glut04 was serum of mice previously successfully immunised with recombinant HMW Dy10 by Dewar (2009) as part of his MD thesis.

The coating concentrations had been worked out in earlier experiments, not shown in this thesis. Serum from the first group of mice immunised in Chapter 5 was tested at different coating concentrations ranging from 6.25µg/ml to 100µg/ml. The proteins were diluted in carbonate buffer, PBS and 60% ethanol each used at these different concentrations. The optimum coating antigen concentration was able to detect the samples and the positive control of previously immunised Dy10 immunised mouse serum as well as having a low background optical density.

The protocol is described in brief below; all steps were carried out at 37°C:

1. 100µl of sample or control serum was added and underwent doubling dilutions before incubated for 45 minutes

2. Anti-mouse IgG-alkaline phosphatase antibody produced in rabbit was added (1 in 2000, A4312, Sigma) and incubated for 45 minutes
3. 100µl of alkaline phosphatise yellow (pNPP) liquid substrate (P7998, Sigma) was added and incubated in the dark for 20-30 minutes.

The plate was washed with PBS/0.05% v/v Tween 20 (T9039, Sigma) after step 1 and 2. The optical density of each sample was determined using an ELISA reader (Biotec ELx800) with the wavelength set at 405nm.

A positive titre was taken as a result greater than 1:6400 as previous experiments done by the laboratory group have shown that this titre is required for successful hybridoma formation (Ellis 1989).

2.7.6 Splenocyte harvesting

Mice that had a good serological response to the immunogen were given an intravenous injection of the candidate peptide (50µg dissolved in 100µl 0.9% saline) 2 to 10 weeks after the tail bleed. Three to five days after the intravenous injection the mice were culled by cervical dislocation. Spleens were blunt dissected from the cadaver after drenching the fur with 100% v/v 74OP ethanol to sterilise. They were immediately placed in 5ml double strength antibiotic medium (appendix II) for 30 minutes. The spleens were rinsed twice in 5ml single strength antibiotic medium (appendix II) before resuspending in 2ml single strength antibiotic medium and disrupted using a manual tissue homogeniser to remove the splenic capsule. The liquid was centrifuged at 1500rpm (300g) for ten minutes. Five millilitres of red blood cell lysing buffer (R7757, Sigma) was mixed with the dry pellet to remove the erythrocytes from the splenocytes and under-layered with 2ml foetal calf serum (FCS). This was centrifuged at 15000rpm (300g) for 10 minutes and the pellet was resuspended in 5ml serum-free medium. At least three washes with 15ml RPMI was required to remove the FCS. This was deemed clean when no bubbles were visible on gentle agitation of the centrifuge tube.

During the stage of blunt dissection, serum was also obtained by opening the thoracic cavity, transecting the heart and collecting the pooled blood before it had a chance to clot using a small syringe, with subsequent centrifugation at 13000rpm (10,000g) for 5 minutes using a benchtop centrifuge (MSE MicroCentaur) and discarding the clot. Serum was then frozen at -20 ºC for use in screening hybridomas and as a positive control for future screening experiments.

2.7.7 Fusion of splenocyte-myeloma hybridomas

Splenocytes cannot be maintained in culture. If they are fused with cancer cells, myeloma in this case (P3X63Ag8.653, Health Protection Agency Culture Collections), they produce hybridoma cells that have the immortal qualities of the cancer cell with the antibody production properties of the splenocyte.

An average mouse spleen was assumed to have $1x10^8$cells. The optimal ratio of splenocytes to myeloma cells is 2-10:1 therefore the number of myeloma cells required per spleen is in the range of $1\text{-}10x10^7$. A 1% aliquot of the myeloma cells were kept in a separate well to use as an unfused myeloma cell control well. Splenocytes were added to serum-free myeloma cells and the pellet was dried by centrifugation at 15000rpm (300g) for 10 minutes and then carefully removing all medium. These cells were agitated to loosen the pellet before the addition of polyethylene glycol.

Polyethylene glycol (P7181, Sigma) was used to reduce surface tension allowing the membranes to fuse. After 2 minutes on ice, the fusion process was terminated by gentle addition of 1ml of medium with constant shaking and further slow addition of 9ml complete medium and centrifugation at 15000rpm (300g) for 10 minutes. This process was repeated to remove all traces of PEG as this inhibits cell proliferation. The cell pellet was then resuspended in 100ml hypoxanthine aminopterin and thymidine (HAT) medium (H0262, Sigma) (appendix II) and plated into four 24 flat bottomed well plate (Nunc) with one well dedicated to the control, unfused myeloma cells. Each well was then topped up with more HAT medium to 2ml.

2.7.8 Hybridoma culture

Cells were screened after 5-7 days. After fusion the selection of viable cells in culture are as follows, unfused cells, myeloma cells fused to myeloma cells, splenocytes fused to splenocytes and the hybridomas. Splenocytes normally die within 5-7 days in medium whereas myeloma cells proliferate rapidly. Hybridoma cells are vulnerable after the first few days of fusion and can be overwhelmed by the myelomas cells. To ensure this did not happen HAT medium is used.

Cells produce DNA by either *de-novo* pathways or by salvage pathways, using preformed bases in either HAT or hypoxanthine thymidine (HT, H0137, Sigma) medium. Aminopterin is a folic acid analogue which inhibits the enzyme dihydrofolate reductase, critical in the

de-novo synthesis of DNA. Therefore in HAT medium, cells can only survive if they are able to utilize the salvage pathways for DNA synthesis with the preformed bases provided by thymidine and hypoxanthine in the medium. Splenocytes are able to provide the salvage pathway and therefore in the HAT medium only the hybridoma cells are able to survive.

Once the myeloma cells in the control well are dead it was assumed that the unfused myeloma cells in the hybridoma cultures are also dead and the medium was changed to HT medium (appendix II). This allowed the hybridomas to grow more rapidly. Two millilitres of the HAT medium is gently removed and replaced with 2ml of HT medium.

2.7.9 Screening of hybridoma colonies

Once the hybridoma colonies were of sufficient size the supernatant was carefully removed so as not to disturb the colony. This was screened by ELISA for the presence of antibodies using the following protocol:

1. 100µl of supernatant from the well the hybridoma colonies were grown in was added to 20µl Hepes (H0887, Sigma)
2. ELISA plates were coated with PT Dy10 3.8µg/ml in carbonate buffer for HMW $glut_{04}$ hybridoma colonies or LMW glt_{156} MAP 50µg/ml in 60% ethanol for LMW glt_{156} colonies and incubated overnight at 4°C.
3. They were blocked with 1% BSA (A7030, Sigma) at 37°C for an hour.
4. 50µl of supernatant was added to the ELISA plate and incubated for 45 minutes at 37°C
5. Anti-mouse IgG-alkaline phosphatase antibody produced in rabbit was added (1 in 1000, A4312, Sigma) or Anti-mouse IgM-alkaline phosphatase antibody produced in goat (1 in 1000, A9688, Sigma) and incubated for 45 minutes at 37°C
6. 100µl of alkaline phosphatise yellow (pNPP) liquid substrate (P7998, Sigma) was added and incubated in the dark for 20-30 minutes.

The plate was washed with PBS/1%v/v Tween 20 three times after steps 2-6. The optical density of each sample was determined using an ELISA reader (Biotec ELx800) with the wavelength set at 405nm.

2.7.10 Immunoglobulin precipitation

The supernatant from the hybridoma colony was collected in order to precipitate the antibodies in suspension. This allowed the antibodies to be purified and concentrated.

Precipitation works on the principle that the solubility of the antibodies in the medium is altered when a saturated solution of ammonium sulphate (A4915, Sigma) is added to the medium allowing them to come out of solution and be collected by centrifugation. The following method was used to extract the antibodies from the hybridoma supernatant.

The starting volume of supernatant was measured and noted for each sample. An equal volume of saturated ammonium sulphate solution was added dropwise while mixing with a magnetic stirrer. The solution was left for a minimum of 6 hours in the fridge and then centrifuged at 3000rpm (1200g) for 30 minutes. The pellet was resuspended in PBS at a tenth of its original supernatant volume. This was then added to dialysis tubing (molecular weight cut off 12500 Daltons, D9402, Sigma) and dialysed against 3 changes of PBS for 1hr each time to remove excess salts.

Chapter 3: Immunogenicity of glutenins in adaptive immunity

3.1 Introduction and aims

Until recent years, glutenins could not be extracted in pure form without gliadin contamination.They were therefore erroneously assumed not to stimulate the small intestines of coeliac patients. However, work from van de Wal (1999), Vader (2002a), Molberg (2003), Dewar (2006) and Ellis (2006) suggests that glutenins are immunostimulatory in coeliac disease. The epitopes contributing to this response are not yet known.

Van de Wal *et al* tested different peptide sequences, 18 amino acids in length, spanning the entire high molecular weight glutenin (HMWG) molecule with a single T-cell clone from a paediatric human leucocyte antigen (HLA)-DQ8 restricted coeliac patient. The group was able to demonstrate that the minimal epitope required for optimal T-cell stimulation was residues 724-734, QGYYPTSPQQS. Vader *et al* tested T-cell clones of children with coeliac disease with an array of proteins and peptides; some were already known to be toxic whilst others, including the peptide low molecular weight glutenin 156 (LMW glt_{156}), were novel. These clones were grown from twenty-five children newly diagnosed with coeliac disease producing twenty-eight T-cell lines, only four of which were gluten-sensitive after a week in culture. These four gluten-sensitive T-cell lines were cloned and used in his experiments. Four out of sixteen T-cell clones from these children were sensitive to LMW glutenin 156. None of the 4 adult coeliac T-cell lines from small intestinal biopsies reacted to this peptide. The drawback in both these studies is that the reaction with T-cell clones is too specific and unlikely to represent the mass action of T-cells present in the gut (see section 1.4).

We still know little about the toxicity of the HMW subunits known as 1Dx5, 1Bx7, 1By9 and 1Dy10. Dewar *et al* demonstrated the immunostimulatory potential of HMW glutenins by demonstrating *in vitro* stimulation of T-cell lines in eleven of seventeen coeliac patients with a mixture of chemically purified Dx5, Bx7, By9 and Dy10 sub-units of HMW glutenin (see section 1.9.2). They also performed *in vivo* challenges in three patients with coeliac disease taking small intestinal biopsies every hour for six consecutive hours after instillation of the chemically purified HMW glutenin into the duodenum (Dewar 2009). All

patients developed significant change in intestinal morphology after 4hrs. Molberg *et al* (2003) managed to stimulate 5 out of 12 HLA-DQ2 restricted small intestinal T-cell lines when testing recombinant 1Dy10 and 3 out of 12 HLA-DQ2 restricted small intestinal T-cell lines with recombinant 1Dx5. The toxicity of recombinant 1Dx5 and 1Dy10 fraction of HMWG was further demonstrated *in vitro* where 3 out of 14 and 3 out of 11 small intestinal T-cell lines were stimulated respectively (Ellis 2006), and *in vivo* in studies mentioned earlier with chemically purified fractions (Dewar 2006) and recombinant proteins (Ellis 2006), see section 1.12.

Tye-Din *et al* (2010) made significant progress inunravelling potential immunostimulatory epitopes using peripheral blood lymphocytes in adults. However, the research only included individuals who were HLA-DQ2 positive and excluded HLA-DQ8 positive individuals (see section 1.11). The group tested a large peptide library covering wheat gliadins and glutenins, rye and barley peptide epitopes with oligopeptides 20 amino acids in length following a five-day oral gluten challenge. In his initial screening of peptides he found that both the HMWG and LMWG peptides did not produce large IFN-γ T-cell responses as tested by enzyme-linked immunosorbent spot (ELISPOT). Therefore, the group further tested only one LMWG oligopeptide and 4 HMWG oligopeptides. In Tye-Din's study, in the majority of adult patients tested, the peripheral blood lymphocytes failed to produce interferon-γ. Peripheral blood responses to gluten in CD are thought to be less relevant to the CD pathology than small intestinal T-cell responses. This is highlighted by the fact that some non-coeliac individuals have been shown to produce a blood T-cell response to gluten. Furthermore, responses to peripheral blood lymphocytes can be restricted not only by the disease-associated HLA-DQ2 molecule but are also restricted by HLA-DP and HLA-DR (Gjertsen 1994).

The aim of this study was to assess the adaptive immune system response to two glutenin candidate immunostimulatory epitopes, HMW $glut_{04}$ and LMW glt_{156}. These peptides were assessed by proliferation assays using T-cell lines grown from small intestinal biopsies from coeliac individuals and interferon-γ secretion from these T-cells. The HMW $glut_{04}$ peptide is contained within the Dy10 subunit of HMW glutenin protein and is also found in the HMW glutenin peptide W24 in the Tye-Din study (2011). The LMW glt156 peptide was not reported in Tye-Din's study. The null hypothesis in these experiments is that coeliac small intestinal T-cell lines are not simulated by the candidate epitopes HMW $glut_{04}$ and LMW glt_{156}.

3.2 Methods used

3.2.1 T-cell studies

Small intestinal T-cells were isolated and cultured initially against Frazer's Fraction III (FFIII) and subsequently against peptic-tryptic digest of whole gluten (both antigens 5mg/ml) as described earlier in section 2.3, 2.4.1, 2.4.2 and 2.4.3. The culturing antigen was changed as a result of the animal screening experiments for immunisation of peptides which found a poor response as tested with FFIII as the ELISA coating antigen compared with either the HMW glutenin subunit Dy10 or the multiple antigenic peptide (see section 5.4). A peptic-tryptic digest of whole gluten was felt to be more representative of the spectrum of gluten peptides presented to the small intestine in a gluten challenge.

The lines underwent weekly antigen restimulation and maintenance with IL-2, see section 2.4.4. A proliferation assay was set up to determine gluten specificity and the proliferative response to the peptides as described earlier in section 2.4.5. Phytohaemagglutinin (PHA) was used to assess the cell viability in the assay with tests of T-cells alone and APCs alone, either fresh or thawed but not irradiated. The negative control in the experiment was the T-cells in culture alone with PBMCs to act as the antigen-presenting cells. The positive control used in the assay was intitially FFIII and subsequently changed to a peptic-tryptic digest of industrial whole gluten. This was taken as a marker that the T-cells had retained their antigen specificity. Timing of the testing was dependant on sufficient T-cell numbers being achieved ($1.08x10^6$ T-cells). T-cell lines were not used after three antigen restimulation as it is accepted that this was less likely to be a polyclonal T-cell line.

3.2.2 Interferon-γ analysis

T-cell secretion of interferon-γ in the assay supernatants was measured by ELISA using a commercial kit, according to the manufacturer's instructions (R+D systems, Minneapolis, USA). This method has been described in section 2.5.1.

3.2.3 Statistics

GraphPad Prism 5.0 was used for statistical analysis. A one-way repeated measurement ANOVA with Dunnett's multiple comparison test was used to compare the means of the each candidate group against the control group of T+APC. This was not possible for the non-coeliac control group as there were only two patients analysed.

3.3 Peptides

The rationale for choosing the peptides in this study has already been explained in section 1.12 and in the introduction to this chapter, section 3.1. HMW glut04 peptide is contained in the HMW protein subunit Dy10 and in the HMW glutenin peptide W24 (Tye-Din 2011). The following peptides were made commercially by GenScript (New Jersey, USA) with the minimum stimulating T-cell epitope in bold and the underlined glutamine residues a target for deamidation to glutamic acid:

Amino acids 721-735 of HMW glutenin, HMW glutenin$_{04}$ (HMW glut$_{04}$)

QGQ**QGYYPTSPQ**QSG

Amino acids 44-59 of LMW glutenin LMW glutenin$_{156}$ (LMW glt$_{156}$)

PPFSQQQQSPFSQQQQ

3.4 Patients used in T-cell studies

Most cell lines used in this study were freshly grown from patients with a diagnosis of coeliac disease (SD patient codes). A few frozen cell lines were also used (JE patient codes) with their frozen peripheral blood mononuclear cells and stored serum. These cell lines were known to be Frazer's Fraction III (FFIII) sensitive having been used in previous manuscripts (Ellis 2005, Dewar 2006). They had been stored in liquid nitrogen for between 7-9 years and had serum stored at -20°C. When these cell lines were defrosted, they were grown against either FFIII or peptic- tryptic digest of gluten (PT gluten) for a week (see section 2.4.4) before being used in a proliferation assay (see section 2.4.5). Unfortunately, there was limited data from the HLA status of these patients as it was not routine practice in the unit at that time to measure this. Two cell lines were grown from patients who were not coeliac in order to compare responses to the glutenin peptides.

The demographic details of the patients used in this study are shown in Tables 3.1 for cells grown against FFIII and 3.2 for cells grown against petic-tryptic digest of whole gluten. The length of time a patient had coeliac disease was taken as the time from their first diagnostic small intestinal biopsy to the time for the study small intestinal biopsy. There are only a few HLA DQ8 positive patients in these studies which reflects the prevalence in the coeliac population.

Table 3.1 Demographic details of patients whose T-cells were grown against Frazer's Fraction III

Patient	Age (years)	Sex	HLA status	Length of time with coeliac disease	Other diagnoses
SD17	22	M	DQ2	4 months	nil
SD18	35	F	DQ2.5	1 year	nil
JE2	NA	NA	NA	NA	NA
SD20	22	F	DQ8	1 year	nil
SD21	47	F	NA	17 years	Bowel cancer
JE6	NA	NA	NA	NA	NA
JE8	NA	NA	NA	NA	NA
JE9	NA	NA	NA	NA	NA
JE10	NA	NA	NA	NA	NA
SD33	46	F	DQ2	10 years	IBS

NA not available. SD patient codes refer to freshly isolated cells whereas JE patient codes were T-cell lines grown against FFIII and stored in liquid nitrogen for between 7-9 years. For the frozen cell lines, limited information was available. Length of time with coeliac disease refers to the length of time from diagnosis to the small intestinal biopsies taken for the study.

Table 3.2. Demographic details of patients whose T-cells were grown against peptic-tryptic digested gluten

Patient	Age (years)	Sex	HLA status	Length of time with coeliac disease	Other diagnoses
SD35	49	F	DQ2	8 years	osteoarthritis
SD36	61	F	DQ2	1 year	dermatitis herpetiformis
SD33	46	F	DQ2	10 years	IBS
SD38	28	M	DQ2	6 years	nil
SD39	46	F	DQ2.5	6 month	hypothyroid, recurrent miscarriages
JE11	NA	NA	NA	NA	NA
JE13	NA	NA	NA	NA	NA
SD42	31	F	DQ2/8	5 months	type 1 DM
SD45	66	F	DQ2	17 years	hypothyroid, IBS
SD46	57	M	DQ2	2 years	SBBO
SD48	38	M	NA	non-coeliac	GORD, hypercholesterolaemia
SD50	59	F	DQ2	12 years	psoriasis, meningioma resected
SD51	26	F	DQ8	9 years	nil
SD52	46	F	DQ2	5 months	nil
SD56	69	F	DQ2	7 years	microscopic colitis, SBBO, IBS
SD57	73	F	DQ2	22 years	microscopic colitis, hypothyroid, Barrett's, IBS, eczema
SD58	36	F	DQ2	2 years	IBS
SD59	66	F	DQ2	12 years	type 1 DM
SD61	39	F	DQ2	2 years	osteopenia
SD62	24	F	DQ5/6	non-coeliac	IgA deficiency, IBS
SD63	47	F	DQ2	4 years	SLE, recurrent GU
SD64	63	F	DQ2	25 years	nil
SD65	44	F	DQ2	6 years	SBBO
SD66	56	F	DQ2	5 months	osteopenia, asthma
SD68	65	F	DQ2/8	6 years	nil
SD69	31	M	DQ2	5 months	GORD, hiatus hernia
SD71	54	F	DQ2	9 years	nil
SD72	68	F	DQ2	15 years	hypothyroid, IHD, OA, lap cholecystectomy
SD73	57	F	DQ2	4 months	nil
SD33T	48	F	DQ2	12 years	IBS

NA not available. SD patient codes refer to freshly isolated cells whereas JE patient codes were T-cell lines grown against FFIII and stored in liquid nitrogen for between 7-9 years. For the frozen cell lines, limited information was available. Length of time with coeliac disease refers to the length of time from diagnosis to the small intestinal biopsies taken for the study. Patients in grey are non-coeliac control patients.

3.5 Results

3.5.1 Result of Frazer's Fraction III studies

Initially, T-cells were grown against Frazer's Fraction III (FFIII), as a soluble gluten antigen that may possibly contain glutenins. ELISA plates coated with FFIII to assess serological response to the glutenin peptides in mice suggested that possibly these epitopes were not found in FFIII (see section 5.4). The results obtained from FFIII stimulation of coeliac small intestinal T-cells in proliferation assay are summarised in table 3.3, with individual patient results shown in tables 3.4-3.13. The stimulation index (SI) is calculated by dividing the mean counts per minute (CPM) of the candidate peptide by the value obtained for the corresponding control assay containing no antigen. A response of 2 or greater was considered to be positive, as in previous proliferation study literature (Molberg 2001, Vader 2002a, Molberg 2003, Dewar 2006).

The majority of T-cell lines tested against FFIII failed to produce a T-cell response against the positive control, let alone any candidate peptides. During this assay it was clear that the T-cells were not at their optimum with a poor stimulation with phytohaemaggluttinin (PHA), especially with the frozen T-cell lines, whereas the PBMCs were well stimulated by the PHA, as shown in tables 3.4-3.13. Unfortunately the PHA control was added in to screen for problems with the assay during the development of the assay and therefore not all tests contained PHA.

Interferon-γ measurement in the culture supernatants was introduced as another assessment of immunostimulation towards the end of these assays. In the last patient SD33, see table 3.13, there was a four-fold augmentation of the IFN-γ secretion indicating that the cell line was gluten-sensitive but no response to the candidate peptides.

Table 3.3. Results of T-cell proliferation assays against Frazer's Fraction III, high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

Patient number	Weeks in culture	HLA	**T-cells + APC no antigen**	**T-cells + APC with Frazer's Fraction III**		**T-cells + APC with HMW glut$_{04}$**		**T-cells +APC with LMW glt$_{156}$**		**T-cells +APC with deamidated LMW glt$_{156}$**	
			Mean CPM ±SD	Mean CPM ±SD	SI	Mean CPM ±SD	SI	Mean CPM ±SD	SI	Mean CPM ±SD	SI
SD17	2	DQ2	98±42	202±206	2.06	260±207	2.60	115±47	1.17	136±44	1.37
SD18	2	DQ2.5	169±21	149±11	1.10	135±18	0.69	129±18	0.76	262±127	1.55
JE2	3	NA	126±3	95±56	0.75	132±12	1.05	155±10	1.23	121±9	0.96
SD20	1	DQ2/8	365±87	476±169	1.33	192±51	0.52	331±116	0.91	1739±1413	4.7
SD21	2	NA	237±27	243±33	1.02	179±37	0.76	202±33	0.85	273±74	1.15
JE6	3	NA	327±127	754±110	2.3	247±161	0.76	319±223	0.98	305±250	0.93
JE8	3	NA	6740±1671	2158±1224	0.32	7880±885	1.17	8616±1108	1.28	8051±611	1.19
JE9	3	NA	139±17	167±17	1.2	180±15	1.29	173±24	1.66	171±18	1.23
JE10	3	NA	229±4	309±108	1.23	333±74	1.45	365±35	1.6	326±21	1.43
SD33	2	DQ2	145±18	862±182	5.94	145±12	1.00	112±15	0.77	102±9	0.70

NA indicates those patients in whom HLA status was not tested. SD patient codes refer to freshly isolated cells whereas JE patient codes were T-cell lines grown against Frazer's Fraction III (FFIII) and stored in liquid nitrogen for between 7-9 years. T-cells were isolated from small intestinal biopsies from patients with coeliac disease. The lymphocytes were isolated and cultured in the presence of interleukin-2 and weekly antigenic restimulation with FFIII. When $1.08x10^6$ cells were present, autologous irradiated peripheral blood mononuclear cells were pre-incubated with the relevant antigen to act as antigen-presenting cells and proliferation assays were set up to measure the T-cells proliferation response to various antigens. Results were calculated as the stimulation index; the mean incorporation of tritiated thymidine expressed as counts per minute (CPM) for T-cells plus antigen-presenting cells with antigen divided by the mean CPM for these cells without antigen.

Table 3.4 Results of SD17 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ**
T-cells + APC no antigen	98±42	1	NA
T-cells alone with PHA	289±71	2.94	NA
APC alone with PHA	NA	NA	NA
T-cells + APC with Fraser's Fraction III	202±206	2.06	NA
T-cells + APC with HMW glut$_{04}$	260±207	2.06	NA
T-cells + APC with LMW glt$_{156}$	166±48	1.17	NA
T-cells + APC with deamidated LMW glt$_{156}$	135±44	1.37	NA

T-cell proliferation assay result for SD 17 T-cells (HLA DQ2). They spent 2 weeks in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.5 Results of SD18 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ**
T-cells + APC no antigen	169±21	1	NA
T-cells alone with PHA	170±12	1	NA
APC alone with PHA	NA	NA	NA
T-cells + APC with Fraser's Fraction III	149±11	0.88	NA
T-cells + APC with HMW glut$_{04}$	135±18	0.80	NA
T-cells + APC with LMW glt$_{156}$	129±18	0.76	NA
T-cells + APC with deamidated LMW glt$_{156}$	262±127	0.75	NA

T-cell proliferation assay result for SD18 T-cells (HLA DQ2.5) They spent 2 weeks in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.6 Results of JE2 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ**
T-cells + APC no antigen	126±3	1	NA
T-cells alone with PHA	139±37	1.1	NA
APC alone with PHA	30674±1978	167	NA
T-cells + APC with Fraser's Fraction III	95±56	0.75	NA
T-cells + APC with HMW glut$_{04}$	132±12	1.05	NA
T-cells + APC with LMW glt$_{156}$	155±10	1.23	NA
T-cells + APC with deamidated LMW glt$_{156}$	121±9	0.96	NA

T-cell proliferation assay result for JE2 T-cells (HLA DQ status not available). This was a frozen cell line which spent 3 weeks in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.7 Results of SD20 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ**
T-cells + APC no antigen	365±87	1	NA
T-cells alone with PHA	457±53	1.25	NA
APC alone with PHA	2646±1866	7.25	NA
T-cells + APC with Fraser's Fraction III	476±169	1.31	NA
T-cells + APC with HMW glut$_{04}$	192±51	0.53	NA
T-cells + APC with LMW glt$_{156}$	331±116	0.91	NA
T-cells + APC with deamidated LMW glt$_{156}$	1739±1413	4.76	NA

T-cell proliferation assay result for SD20 T-cells (HLA DQ8). They spent a 1 week in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.8 Results of SD21 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ**
T-cells + APC no antigen	237±27	1	NA
T-cells alone with PHA	236±123	1	NA
APC alone with PHA	63045±8860	266	NA
T-cells + APC with Fraser's Fraction III	243±33	1.03	NA
T-cells + APC with HMW glut$_{04}$	179±37	0.76	NA
T-cells + APC with LMW glt$_{156}$	210±33	0.85	NA
T-cells + APC with deamidated LMW glt$_{156}$	273±74	1.15	NA

T-cell proliferation assay result for SD21 T-cells (HLA DQ status not available). They spent 2 weeks in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.9 Results of JE6 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ**
T-cells + APC no antigen	327±127	1	NA
T-cells alone with PHA	1150±101	3.51	NA
APC alone with PHA	24978±1881	76.31	NA
T-cells + APC with Fraser's Fraction III	754±110	2.30	NA
T-cells + APC with HMW glut$_{04}$	248±161	0.76	NA
T-cells + APC with LMW glt$_{156}$	320±223	0.98	NA
T-cells + APC with deamidated LMW glt$_{156}$	302±250	0.93	NA

T-cell proliferation assay result for JE6 T-cells (HLA DQ status not available). This was a frozen cell line which spent 3 weeks in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.10 Results of JE8 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ**
T-cells + APC no antigen	6740±1671	1	NA
T-cells alone with PHA	3848	0.58	NA
APC alone with PHA	2291	0.44	NA
T-cells + APC with Fraser's Fraction III	2158±1225	0.33	NA
T-cells + APC with HMW glut$_{04}$	7881±885	1.17	NA
T-cells + APC with LMW glt$_{156}$	8617±1108	1.28	NA
T-cells + APC with deamidated LMW glt$_{156}$	8051±612	1.19	NA

T-cell proliferation assay result for JE8 T-cells (HLA DQ status not available). This was a frozen cell line which spent 3 weeks in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. Where there is no standard deviation result there were too few cells for triplicate assays and a single assay result was used. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.11 Results of JE9 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ**
T-cells + APC no antigen	139±17	1	NA
T-cells alone with PHA	141±20	1.01	NA
APC alone with PHA	88±14	0.63	NA
T-cells + APC with Fraser's Fraction III	167±17	1.20	NA
T-cells + APC with HMW glut$_{04}$	180±16	1.29	NA
T-cells + APC with LMW glt$_{156}$	173±24	1.24	NA
T-cells + APC with deamidated LMW glt$_{156}$	171±18	1.23	NA

T-cell proliferation assay result for JE9 T-cells (HLA DQ status not available). This was a frozen cell line which spent 3 weeks in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.12 Results of JE10 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ**
T-cells + APC no antigen	229±4	1	NA
T-cells alone with PHA	244±3	1.06	NA
APC alone with PHA	37024±3141	161.89	NA
T-cells + APC with Fraser's Fraction III	309±108	1.35	NA
T-cells + APC with HMW glut$_{04}$	333±74	1.45	NA
T-cells + APC with LMW glt$_{156}$	365±35	1.59	NA
T-cells + APC with deamidated LMW glt$_{156}$	326±21	1.42	NA

T-cell proliferation assay result for JE10 T-cells (HLA DQ status not available). This was a frozen cell line which spent 3 weeks in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.13 Results of SD33 T-cell proliferation assays against Frazer's Fraction III (FFIII), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	135±18	1	20
T-cells alone with PHA	No PHA added	No PHA added	NA
APC alone with PHA	No PHA added	No PHA added	NA
T-cells + APC with Fraser's Fraction III	714±182	5.29	78
T-cells + APC with HMW glut$_{04}$	135±12	1.00	53
T-cells + APC with LMW glt$_{156}$	132±15	0.98	13
T-cells + APC with deamidated LMW glt$_{156}$	120±9	0.89	2

T-cell proliferation assay result for SD33 T-cells (HLA DQ2) They spent 2 weeks in culture grown against Frazer's Fraction III (FFIII). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for FFIII and phytohaemagglutinin, a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

3.5.2 Result of peptic-tryptic digest of whole gluten studies

3.5.2.1 Results from coeliac patients

The results from the Frazer's Fraction III assays did not demonstrate gluten sensitivity, which may be due to poor T-cell survival. Therefore, an additional washing step was added after the biopsy was minced (see section 2.4.3) to minimise the amount of antigen still left in the culture.

A peptic-tryptic digest of whole gluten was subsequently used as the antigen against which the T-cells were grown. This was felt to be more representative of the gluten peptide exposure of T-cells in the small intestine of coeliac patients. Interferon-γ (IFN-γ) concentrations were measured by ELISA and the net excess of interferon-γ secretion is shown. This was obtained by subtracting the value obtained in the negative control assay. A level of 0 indicates there was not an excess of interferon-γ in the test assay compared to the control. NA indicates those patients in whom supernatants could not be tested. The results of the proliferation assays, including IFN-γ concentrations, using these T-cells are shown in table 3.14, spread over 3 pages, with the results of the individual assays shown in tables 3.15-3.42.

Tables 3.15-3.42 show that these glutenin peptides are not major T-cell epitopes with only three of the cell lines, SD35, SD 38 and SD73, achieving positive stimulation indices (SIs). Unfortunately there is a lack of interferon-γ data for these patients. What is interesting is the difference in stimulation indices, SD35 has very high SI to both PT gluten and HMW glut04 while SD 38 and SD73 have more modest SIs towards all the peptides for SD38 and only HMW glut04 for SD73.

It is also interesting that there is little rise in interferon-γ in SD38 in the LMW glt156 and dLMWglt156 compared to the PT gluten response despite similar values for the SI. There is also evidence in other patients, SD 39 and SD33T, for greater interferon-γ secretion towards HMW glut04 with a negative SI, suggesting high secretion of INF-γ from a few T-cells.

The HLA status of the patients unfortunately was difficult to analyse as there were too few patients in this study to allow a proper assessment. However, the two heterzygotes, SD42 and SD68, as well as SD51, the HLA DQ8 homozygote, did not seem to mount an immune response to these peptides.

Certainly SD38 and SD73 were only a week in culture and therefore are more likely to be more representative of the mass T cell action in the gut. Other T cells lines with the same length of time in culture, SD 33 and SD 36, did not react to the HMW glut04 or LMW glt156 whether it was deamidated or not. It did not seem to matter the length of time of diagnosis nor the HLA DQ status in these patients.

Table 3.14 Results of T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59. **Table continues overleaf....**

Pt no	Wk in culture	HLA	**T-cells + APC no antigen**		**T-cells + APC with PT gluten**			**T-cells + APC with HMW glut$_{04}$**			**T-cells +APC with LMW glt$_{156}$**			**T-cells +APC with deamidated LMW glt$_{156}$**		
			Mean CPM ±SD	IFN -γ	Mean CPM±SD	SI	IFN -γ	Mean CPM±SD	SI	IFN -γ	Mean CPM±SD	SI	IFN -γ	Mean CPM±SD	SI	IFN -γ
SD 35	2	DQ2	515±8	NA	1042±236	2.03	NA	9880±1661	19.2	NA	10816±360	21.02	NA	225±110	0.44	NA
SD 36	1	DQ2	154±16	NA	507±85	3.29	NA	66±25	0.43	NA	133±36	0.86	NA	225±25	1.02	NA
SD38	1	DQ2	139± 40	3	2478±698	17.82	123	582±571	4.20	130	419±200	3.01	23	494±193	3.55	32
SD33	1	DQ2	1776±69	13	7354	4.14	93	2914±1580	1.64	13	2038±566	1.15	13	1699±175	0.96	8
SD39	3	DQ2.5	1884±1667	33	8175±288	4.34	640	2649±312	1.41	123	3281±302	1.74	13	3440±158	1.83	27
JE 11	3	NA	189±43	0	717±42	3.8	13	224±36	1.19	2	205±1	1.09	0	248±9	1.31	1
JE 13	3	NA	70±36	NA	861±213	12.38	NA	99±30	1.43	NA	79±7	1.21	NA	196±1	2.82	NA
SD42	2	DQ2/8	904±480	50	2124±261	2.56	110	459±44	0.51	0	485±32	0.54	0	535±35	0.59	35
SD45	2	DQ2	1028±69	10	2128±163	2.07	510	917±205	0.89	0	709±378	0.69	18	783±23	0.76	12
SD46	2	DQ2	128±48	0	329±63	2.56	430	126±36	0.98	0	127±38	0.99	0	139±13	1.08	0
SD50	2	DQ2	322±36	0	4570±179	14.18	340	379±53	1.18	0	391±60	1.12	0	485±60	1.51	0

Table continued overleaf

Table 3.14 cont from previous page: Results of T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59. **Table continues overleaf.....**

Pt no	Wk in culture	HLA	**T-cells + APC no antigen**		**T-cells + APC with PT gluten**			**T-cells + APC with HMW glut$_{04}$**			**T-cells +APC with LMW glt$_{156}$**			**T-cells +APC with deamidated LMW glt$_{156}$**		
			Mean CPM ±SD	IFN-γ	Mean CPM ±SD	SI	IFN-γ	Mean CPM ±SD	SI	IFN-γ	Mean CPM ±SD	SI	IFN-γ	Mean CPM±SD	SI	IFN-γ
SD51	2	DQ8	5776± 1619	0	11497± 2717	2.00	140	6693± 2716	1.16	0	5132 ± 3540	0.89	0	9406±3047	1.63	0
SD52	2	DQ2	218±45	5	3368± 233	15.46	144	316±2	1.45	0	191± 107	0.87	0	335±292	1.54	14
SD56	1	DQ2	2117± 420	12	6330± 213	2.99	100	2118± 113	1	0	2600 ± 270	1.23	0	2880±2	1.36	0
SD57	2	DQ2	14302± 612	53	60355± 1430	4.22	478	14874± 548	1.04	28	10870 ±492	0.76	7	13873± 1720	0.97	NA
SD58	2	DQ2	259±71	0	3046± 1470	11.78	98	275±25	1.06	0	264± 22	1.02	0	224±83	0.86	0
SD59	1	DQ2	2515± 547	0	32750± 888	13.04	98	3428± 1370	1.36	0	3097 ± 197	1.23	0	1577± 377	0.63	0
SD61	2	DQ2	864± 401	0	2532± 449	2.93	103	656±61	0.76	0	503± 51	0.58	0	744± 150	0.86	0
SD63	3	DQ2	422±54	0	3608± 432	8.56	220	436±28	1.03	0	580± 48	1.38	0	704±219	1.67	0
SD64	1	DQ2	702±81	0	4367± 320	6.22	180	814± 260	1.16	0	731± 28	1.04	0	917± 216	1.31	0
SD65	2	DQ2	810± 154	0	2861± 88	3.53	203	875±38	1.08	0	867± 100	1.07	0	1190±1083	1.47	0

Table continued overleaf

Table 3.14 cont from previous page: Results of T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

Pt no	Wk in culture	HLA	**T-cells + APC no antigen**		**T-cells + APC with PT gluten**			**T-cells + APC with HMW glut$_{04}$**			**T-cells +APC with LMW glt$_{156}$**			**T-cells +APC with deamidated LMW glt$_{156}$**		
			Mean CPM ±SD	IFN-γ	Mean CPM ±SD	SI	IFN-γ	Mean CPM ±SD	SI	IFN-γ	Mean CPM ±SD	SI	IFN-γ	Mean CPM ±SD	SI	IFN-γ
SD66	2	DQ2	255±25	0	617±50	2.42	180	299±23	1.17	0	257±13	1.01	0	261±23	1.02	0
SD68	2	DQ2/8	310±49	0	3085±111	9.94	98	425±70	1.37	0	353±79	1.14	0	419±68	1.35	0
SD69	2	DQ2	407±54	0	1095±167	2.69	400	462±31	1.13	0	288±38	0.71	0	364±28	0.9	0
SD71	2	DQ2	218±48	0	468±16	2.15	130	236±31	1.09	0	223±13	1.02	0	237±19	1.09	0
SD72	2	DQ2	961±38	NA	3737±234	3.89	NA	1088±156	1.13	NA	1028±281	1.07	NA	1068±138	1.11	NA
SD73	1	DQ2	2091±88	0	6211±179	5.95	160	4433±839	2.12	18	3392±605	1.62	0	2992±1251	1.43	0
SD33T	2	DQ2	135±18	13	714±182	5.3	78	135±12	1	53	120±6	0.89	13	120±9	0.89	2

NA indicates those patients in whom HLA status was not tested. SD patient codes refer to freshly isolated cells whereas JE patient codes were T-cell lines grown against Frazer's Fraction III (FFIII) and stored in liquid nitrogen for between 7-9 years. T-cells were isolated from small intestinal biopsies from patients with coeliac disease. The lymphocytes were isolated and cultured in the presence of interleukin-2 and weekly antigenic restimulation with peptic-tryptic digest of gluten. When $1.08x10^6$ cells were present, autologous irradiated peripheral blood mononuclear cells were pre-incubated with the relevant antigen to act as antigen-presenting cells and proliferation assays were set up to measure the T-cells proliferation response to various antigens. Results were calculated as the stimulation index; the mean incorporation of tritiated thymidine expressed as counts per minute (CPM) for T-cells plus antigen-presenting cells with antigen divided by the mean CPM for these cells without antigen.

Table 3.15 Results of SD35 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	515±8	1	NA
T-cells alone with PHA	18720±4836	35.8	NA
APC alone with PHA	5171±3290	10.05	NA
T-cells + APC with PT gluten	1042±236	2.03	NA
T-cells + APC with HMW glut$_{04}$	9880±1661	19.2	NA
T-cells + APC with LMW glt$_{156}$	10816±360	21.2	NA
T-cells + APC with deamidated LMW glt$_{156}$	225±110	0.43	NA

T-cell proliferation assay result for SD35 T-cells (HLA DQ2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.16 Results of SD36 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	154±16	1	NA
T-cells alone with PHA	4015±2540	26	NA
APC alone with PHA	2830±1178	18.97	NA
T-cells + APC with PT gluten	507±85	3.28	NA
T-cells + APC with HMW glut$_{04}$	66±25	0.43	NA
T-cells + APC with LMW glt$_{156}$	133±36	0.86	NA
T-cells + APC with deamidated LMW glt$_{156}$	157±25	1.02	NA

T-cell proliferation assay result for SD36 T-cells (HLA DQ2). These spent 1 week in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available

Table 3.17 Results of SD38 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	139±40	1	13
T-cells alone with PHA	2222±140	16.30	223
APC alone with PHA	345870±11290	249.55	1000
T-cells + APC with PT gluten	2478±698	17.88	123
T-cells + APC with HMW glut$_{04}$	582±571	4.2	130
T-cells + APC with LMW glt$_{156}$	419±200	3.3	23
T-cells + APC with deamidated LMW glt$_{156}$	494±193	3.56	32

T-cell proliferation assay result for SD38 T-cells (HLA DQ2). These spent 1 week in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.18 Results of SD33 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	1776±69	1	13
T-cells alone with PHA	202468±32783	114.00	873
APC alone with PHA	40305±40465	22.69	253
T-cells + APC with PT gluten	7354	4.14	93
T-cells + APC with HMW glut$_{04}$	2814±1580	1.63	13
T-cells + APC with LMW glt$_{156}$	2038±566	1.15	13
T-cells + APC with deamidated LMW glt$_{156}$	1699±175	0.96	8

T-cell proliferation assay result for SD33 T-cells (HLA DQ 2). These spent 1 week in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.19 Results of SD39 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	1884±1667	1	33
T-cells alone with PHA	164541±42012	87.35	833
APC alone with PHA	25174±756	13.36	850
T-cells + APC with PT gluten	8175±288	4.34	640
T-cells + APC with HMW glut$_{04}$	2649±312	1.41	123
T-cells + APC with LMW glt$_{156}$	3281±302	1.74	13
T-cells + APC with deamidated LMW glt$_{156}$	3440±158	1.83	27

T-cell proliferation assay result for SD39 T-cells (HLA DQ2.5). These spent 3 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.20 Results of JE11 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	189±43	1	0
T-cells alone with PHA	7830±855	41.51	123
APC alone with PHA	15033±4073	79.69	>1000
T-cells + APC with PT gluten	717±42	3.8	13
T-cells + APC with HMW glut$_{04}$	224±36	1.19	2
T-cells + APC with LMW glt$_{156}$	205±1	1.09	0
T-cells + APC with deamidated LMW glt$_{156}$	248±9	1.31	1

T-cell proliferation assay result for JE11 T-cells (HLA DQ status not available). This was a frozen cell line which spent 3 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.21 Results of JE13 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	70±36	1	NA
T-cells alone with PHA	4608±5097	66.25	NA
APC alone with PHA	8119±5732	116.74	NA
T-cells + APC with PT gluten	861±213	12.38	NA
T-cells + APC with HMW glut$_{04}$	99±30	1.42	NA
T-cells + APC with LMW glt$_{156}$	79±7	1.14	NA
T-cells + APC with deamidated LMW glt$_{156}$	196±1	2.82	NA

T-cell proliferation assay result for JE13 T-cells (HLA DQ status not available). This was a frozen cell line which spent 3 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. NA result not available as supernatants were not removed.

Table 3.22 Results of SD42 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	904±480	1	50
T-cells alone with PHA	9446±585	10.45	>1000
APC alone with PHA	50116±10985	55.45	>1000
T-cells + APC with PT gluten	2124±261	2.58	110
T-cells + APC with HMW glut$_{04}$	459±44	0.51	0
T-cells + APC with LMW glt$_{156}$	485±32	0.54	0
T-cells + APC with deamidated LMW glt$_{156}$	535±35	0.59	35

T-cell proliferation assay result for SD42 T-cells (HLA DQ2/8). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.23 Results of SD45 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	1028±69	1	10
T-cells alone with PHA	58514±5803	56.92	>1000
APC alone with PHA	53149±10133	51.70	>1000
T-cells + APC with PT gluten	2128±163	2.07	510
T-cells + APC with HMW glut$_{04}$	917±205	0.89	0
T-cells + APC with LMW glt$_{156}$	709±378	0.69	18
T-cells + APC with deamidated LMW glt$_{156}$	783±230	0.76	12

T-cell proliferation assay result for SD45 T-cells (HLA DQ2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.24 Results of SD46 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	128±48	1	0
T-cells alone with PHA	833±63	6.48	>1000
APC alone with PHA	823±66	6.04	>1000
T-cells + APC with PT gluten	329±28	2.56	430
T-cells + APC with HMW glut$_{04}$	126±36	0.98	0
T-cells + APC with LMW glt$_{156}$	127±38	0.99	0
T-cells + APC with deamidated LMW glt$_{156}$	139±13	1.08	0

T-cell proliferation assay result for SD46 T-cells (HLA DQ 2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.25 Results of SD50 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	322±36	1	0
T-cells alone with PHA	17753±14960	55.13	810
APC alone with PHA	16207±4364	50.33	760
T-cells + APC with PT gluten	4570±179	14.19	340
T-cells + APC with HMW glut$_{04}$	379±53	1.18	0
T-cells + APC with LMW glt$_{156}$	391±60	1.21	0
T-cells + APC with deamidated LMW glt$_{156}$	485±60	1.51	0

T-cell proliferation assay result for SD50 T-cells (HLA DQ2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.26 Results of SD51 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	5776±1619	1	0
T-cells alone with PHA	35468±23010	6.14	230
APC alone with PHA	48525±71804	8.04	310
T-cells + APC with PT gluten	11497±2717	2	140
T-cells + APC with HMW glut$_{04}$	6693±2716	1.16	0
T-cells + APC with LMW glt$_{156}$	5132±3540	0.89	0
T-cells + APC with deamidated LMW glt$_{156}$	9406±3047	1.63	0

T-cell proliferation assay result for SD51 T-cells (HLA DQ8). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.27 Results of SD52 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	218±45	1	5
T-cells alone with PHA	1100±116	5.05	>1000
APC alone with PHA	19876±4477	91.24	>1000
T-cells + APC with PT gluten	3368±233	15.46	144
T-cells + APC with HMW glut$_{04}$	316±2	1.45	0
T-cells + APC with LMW glt$_{156}$	191±107	0.87	0
T-cells + APC with deamidated LMW glt$_{156}$	335±292	1.54	14

T-cell proliferation assay result for SD52 T-cells (HLA DQ2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells. NA result not available

Table 3.28 Results of SD56 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	2117±420	1	12
T-cells alone with PHA	41904±10295	19.79	>1000
APC alone with PHA	71253±3749	33.65	>1000
T-cells + APC with PT gluten	6330±213	2.99	100
T-cells + APC with HMW glut$_{04}$	2118±113	1	0
T-cells + APC with LMW glt$_{156}$	2600±270	1.23	0
T-cells + APC with deamidated LMW glt$_{156}$	2880±2	1.36	0

T-cell proliferation assay result for SD56 T-cells (HLA DQ2). These spent 1 week in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells. NA result not available

Table 3.29 Results of SD57 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	14302±612	1	53
T-cells alone with PHA	83943±5363	5.86	598
APC alone with PHA	302189±50281	21.13	733
T-cells + APC with PT gluten	60355±1430	4.22	478
T-cells + APC with HMW glut$_{04}$	14874±548	1.04	28
T-cells + APC with LMW glt$_{156}$	10870±492	0.78	NA
T-cells + APC with deamidated LMW glt$_{156}$	13873±1720	0.97	NA

T-cell proliferation assay result for SD57 T-cells (HLA DQ2.6). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells. NA result not available

Table 3.30 Results of SD58 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	259±71	1	0
T-cells alone with PHA	10547±157	40.79	743
APC alone with PHA	45130±10159	174.52	693
T-cells + APC with PT gluten	3046±1470	11.78	98
T-cells + APC with HMW glut$_{04}$	275±25	1.06	0
T-cells + APC with LMW glt$_{156}$	264±22	1.02	0
T-cells + APC with deamidated LMW glt$_{156}$	224±83	0.86	0

T-cell proliferation assay result for SD58 T-cells (HLA DQ 2). These 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.31 Results of SD59 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	2515±547	1	0
T-cells alone with PHA	91308±472	36.3	218
APC alone with PHA	35066±12589	13.94	398
T-cells + APC with PT gluten	32750±888	13.04	98
T-cells + APC with HMW glut$_{04}$	3428±1370	1.36	0
T-cells + APC with LMW glt$_{156}$	3097±197	1.23	0
T-cells + APC with deamidated LMW glt$_{156}$	1577±377	0.63	0

T-cell proliferation assay result for SD59 T-cells (HLA DQ2). These spent 1 week in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.32 Results of SD61 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	864±401	1	0
T-cells alone with PHA	14018±1218	16.22	>1000
APC alone with PHA	32833±2786	37.99	>1000
T-cells + APC with PT gluten	2532±449	2.93	103
T-cells + APC with HMW glut$_{04}$	656±61	0.76	0
T-cells + APC with LMW glt$_{156}$	503±51	0.58	0
T-cells + APC with deamidated LMW glt$_{156}$	744±150	0.86	0

T-cell proliferation assay result for SD61 T-cells (HLA DQ2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.33 Results of SD63 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	422±54	1	0
T-cells alone with PHA	8310	19.72	>1000
APC alone with PHA	17264±12	40.96	505
T-cells + APC with PT gluten	3608±432	8.56	220
T-cells + APC with HMW glut$_{04}$	436±28	1.03	0
T-cells + APC with LMW glt$_{156}$	580±48	1.38	0
T-cells + APC with deamidated LMW glt$_{156}$	704±219	1.67	0

T-cell proliferation assay result for SD63 T-cells (HLA DQ2). These spent 3 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.34 Results of SD64 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	702±81	1	0
T-cells alone with PHA	26995±1500	38.45	>1000
APC alone with PHA	49875±2720	71.05	>1000
T-cells + APC with PT gluten	4367±320	6.22	180
T-cells + APC with HMW glut$_{04}$	814±260	1.16	0
T-cells + APC with LMW glt$_{156}$	731±28	1.01	0
T-cells + APC with deamidated LMW glt$_{156}$	917±216	1.31	0

T-cell proliferation assay result for SD64 T-cells (HLA DQ2). These spent 1 week in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.35 Results of SD65 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	810±154	1	0
T-cells alone with PHA	81832±9922	100.97	858
APC alone with PHA	81672±20254	100.76	997
T-cells + APC with PT gluten	2861±88	3.53	203
T-cells + APC with HMW glut$_{04}$	875±38	1.08	0
T-cells + APC with LMW glt$_{156}$	867±100	1.07	0
T-cells + APC with deamidated LMW glt$_{1568}$	1190±1083	1.47	0

T-cell proliferation assay result for SD65 T-cells (HLA DQ). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.36 Results of SD66 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	255±25	1	0
T-cells alone with PHA	18239±2495	71.56	876
APC alone with PHA	29389±4461	115.31	>1000
T-cells + APC with PT gluten	617±50	2.42	180
T-cells + APC with HMW glut$_{04}$	299±23	1.17	0
T-cells + APC with LMW glt$_{156}$	257±13	1.01	0
T-cells + APC with deamidated LMW glt$_{156}$	261±23	1.02	0

T-cell proliferation assay result for SD66 T-cells (HLA DQ). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.37 Results of SD68 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	310±49	1	0
T-cells alone with PHA	31704±4339	102.16	133
APC alone with PHA	73948±13077	238.29	983
T-cells + APC with PT gluten	3085±111	9.94	98
T-cells + APC with HMW glut$_{04}$	425±70	1.37	0
T-cells + APC with LMW glt$_{156}$	353±79	1.14	0
T-cells + APC with deamidated LMW glt$_{156}$	419±68	1.35	0

T-cell proliferation assay result for SD68 T-cells (HLA DQ2/8). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.38 Results of SD69 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	407±54	1	0
T-cells alone with PHA	3893±604	21.97	>1000
APC alone with PHA	76782±7996	188.64	983
T-cells + APC with PT gluten	1095±167	2.69	400
T-cells + APC with HMW glut$_{04}$	462±31	1.13	0
T-cells + APC with LMW glt$_{156}$	288±65	0.71	0
T-cells + APC with deamidated LMW glt$_{156}$	364±49	0.9	0

T-cell proliferation assay result for SD69 T-cells (HLA DQ2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.39 Results of SD71 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	218±48	1	0
T-cells alone with PHA	4783±578	21.97	>1000
APC alone with PHA	26420±7066	121.38	158
T-cells + APC with PT gluten	468±16	2.15	130
T-cells + APC with HMW glut$_{04}$	236±31	1.09	0
T-cells + APC with LMW glt$_{156}$	223±13	1.02	0
T-cells + APC with deamidated LMW glt$_{156}$	237±19	1.09	0

T-cell proliferation assay result for SD71 T-cells (HLA DQ2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.40 Results of SD72 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	961±38	1	NA
T-cells alone with PHA	53010±13343	55.19	NA
APC alone with PHA	124539±11049	129.65	NA
T-cells + APC with PT gluten	3737±234	3.89	NA
T-cells + APC with HMW glut$_{04}$	1088±156	1.13	NA
T-cells + APC with LMW glt$_{156}$	1028±281	1.07	NA
T-cells + APC with deamidated LMW glt$_{156}$	1068±138	1.11	NA

T-cell proliferation assay result for SD72 T-cells (HLA DQ2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells. NA result not available

Table 3.41 Results of SD73 T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	2091±88	1	0
T-cells alone with PHA	22961±5938	10.99	>1000
APC alone with PHA	47381±15578	22.66	>1000
T-cells + APC with PT gluten	6211±179	5.95	160
T-cells + APC with HMW glut$_{04}$	4433±839	2.12	0
T-cells + APC with LMW glt$_{156}$	3392±605	1.62	0
T-cells + APC with deamidated LMW glt$_{156}$	2992±1251	1.43	0

T-cell proliferation assay result for SD73 T-cells (HLA DQ2.7). These spent 1 week in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.42 Results of SD33T T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	135±18	1	13
T-cells alone with PHA	No	PHA	added
APC alone with PHA	No	PHA	added
T-cells + APC with PT gluten	714±182	5.3	78
T-cells + APC with HMW glut$_{04}$	135±12	1	53
T-cells + APC with LMW glt$_{156}$	120±6	0.89	13
T-cells + APC with deamidated LMW glt$_{156}$	120±9	0.89	2

T-cell proliferation assay result for SD33T T-cells (HLA DQ2). These spent 2 weeks in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells. Unfortunately no PHA was added to the T-cell and APC control wells.

3.5.2.2 Result from non-coeliac patients

A small number of patients who did not have a diagnosis of coeliac disease were also included as negative controls and the results are shown in tables 3.43, 3.44 and 3.45. Unfortunately the HLA status of one of the patients is missing and NA indicates that patient in whom HLA status or supernatants could not be tested.

This study demonstrates that T-cells are not stimulated by peptic-tryptic gluten digest as well as the candidate glutenin peptides. Unfortunately due to ethical constraints more patients were not able to be recruited into this study.

Table 3.43. Results of T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59 in non-coeliac patients.

Pt No	Wk in culture	HLA	**T-cells + APC no antigen**		**T-cells + APC with PT gluten**			**T-cells + APC with HMW glut$_{04}$**			**T-cells +APC with LMW glt$_{156}$**			**T-cells +APC with deamidated LMW glt$_{156}$**		
			Mean CPM ±SD	IFN -γ	Mean CPM±SD	SI	IFN -γ	Mean CPM±SD	SI	IFN -γ	Mean CPM±SD	SI	IFN -γ	Mean CPM±SD	SI	IFN -γ
SD48	1	NA	251±78	0	305±31	1.22	0	249±14	0.99	0	294±20	1.17	0	302±31	1.20	0
SD62	1	DQ5/6	376±26	0	372±29	0.99	0	399±38	1.06	0	402±63	1.07	0	527±58	1.40	0

NA indicates that patient in whom HLA status was not tested. SD patient codes refer to freshly isolated cells. T-cells were isolated from small intestinal biopsies from patients without a diagnosis of coeliac disease. The lymphocytes were isolated and cultured in the presence of interleukin-2 and weekly antigenic restimulation with peptic-tryptic digest of gluten. When $1.08x10^6$ cells were present, autologous irradiated peripheral blood mononuclear cells were pre-incubated with the relevant antigen to act as antigen-presenting cells and proliferation assays were set up to measure the T-cells proliferation response to various antigens. Results were calculated as the stimulation index; the mean incorporation of tritiated thymidine expressed as counts per minute (CPM) for T-cells plus antigen-presenting cells with antigen divided by the mean CPM for these cells without antigen.

Table 3.44 Results of SD48, a non-coeliac patient, T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	251±78	1	0
T-cells alone with PHA	4815±278	19.19	310
APC alone with PHA	45334±16638	180.68	>1000
T-cells + APC with PT gluten	305±31	1.22	0
T-cells + APC with HMW glut$_{04}$	249±14	0.99	0
T-cells + APC with LMW glt$_{156}$	294±20	1.17	0
T-cells + APC with deamidated LMW glt$_{156}$	302±31	1.2	0

T-cell proliferation assay result for non-coeliac SD48 T-cells (HLA DQ status unknown) which were 1 week in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen. Interferon-γ was measured in the combined triplicate culture supernatants using an ELISA kit and the net secretion calculated by subtracting the concentration of interferon-γ in wells containing ASM alone from the test protein or peptide wells.

Table 3.45 Results of SD62, a non-coeliac patient, T-cell proliferation assays against peptic-tryptic digest of whole gluten (PT gluten), high molecular weight glutenin$_{04}$ 721-735 (HMW glut$_{04}$), low molecular weight glutenin glt$_{156}$ 44-59 (LMW glt$_{156}$) and deamidated LMW glt$_{156}$ 44-59.

	Mean CPM ± SD	**SI**	**Interferon-γ (pg/ml)**
T-cells + APC no antigen	376±26	1	0
T-cells alone with PHA	1789±363	4.76	>1000
APC alone with PHA	43154±4725	114.87	>1000
T-cells + APC with PT gluten	372±29	0.99	0
T-cells + APC with HMW glut$_{04}$	399±38	1.06	0
T-cells + APC with LMW glt$_{156}$	402±63	1.07	0
T-cells + APC with deamidated LMW glt$_{156}$	527±58	1.4	0

T-cell proliferation assay result for non-coeliac SD62 T-cells (HLA DQ5/6) which were 1 week in culture grown against peptic-tryptic digest of whole gluten (PT gluten). All tests were set up in triplicates with 48 hour incubation with candidate peptides as well as control protein for PT gluten and phytohaemagglutinin (PHA), a potent lymphocyte stimulator. The mean is taken for the counts per minute after an overnight incubation with tritiated thymidine. Stimulation index (SI) has been calculated by dividing the mean CPM of the candidate peptide or protein by the value obtained for the assay containing no antigen.

3.5.3 Comparison of results from all three patient groups

Figure 3.1 compares all the available results for the cells grown against FFFIII (tables 3.4-3.15), PT gluten coeliac patients (tables 3.15-3.42) and non-coeliac patients (tables 3.44 and 3.45). It demonstrates that the HMW glut04 peptide as well as the LMW glt156 peptide, whether or not it is deamidated, is not immunostimulatory to non-coeliac patients' T-cells grown *in vitro*. Only one patient in the FFIII group mounted an immune response to the HMW glut04 pepitde and one other to the deamidated LMW glt156 peptide.

T-cells grown against PT gluten are gluten-sensitive. However, the T-cells are not stimultaed by either of these glutenin peptides. This is evidenced by a lack of SI response as well as interferon-γ secretion into the culture medium. It does demonstrate a heterogenous T-cell response by each individual coeliac patient.

Figure 3.1 Stimulation Indices and Interferon-γ results for tables 3.3-3.45

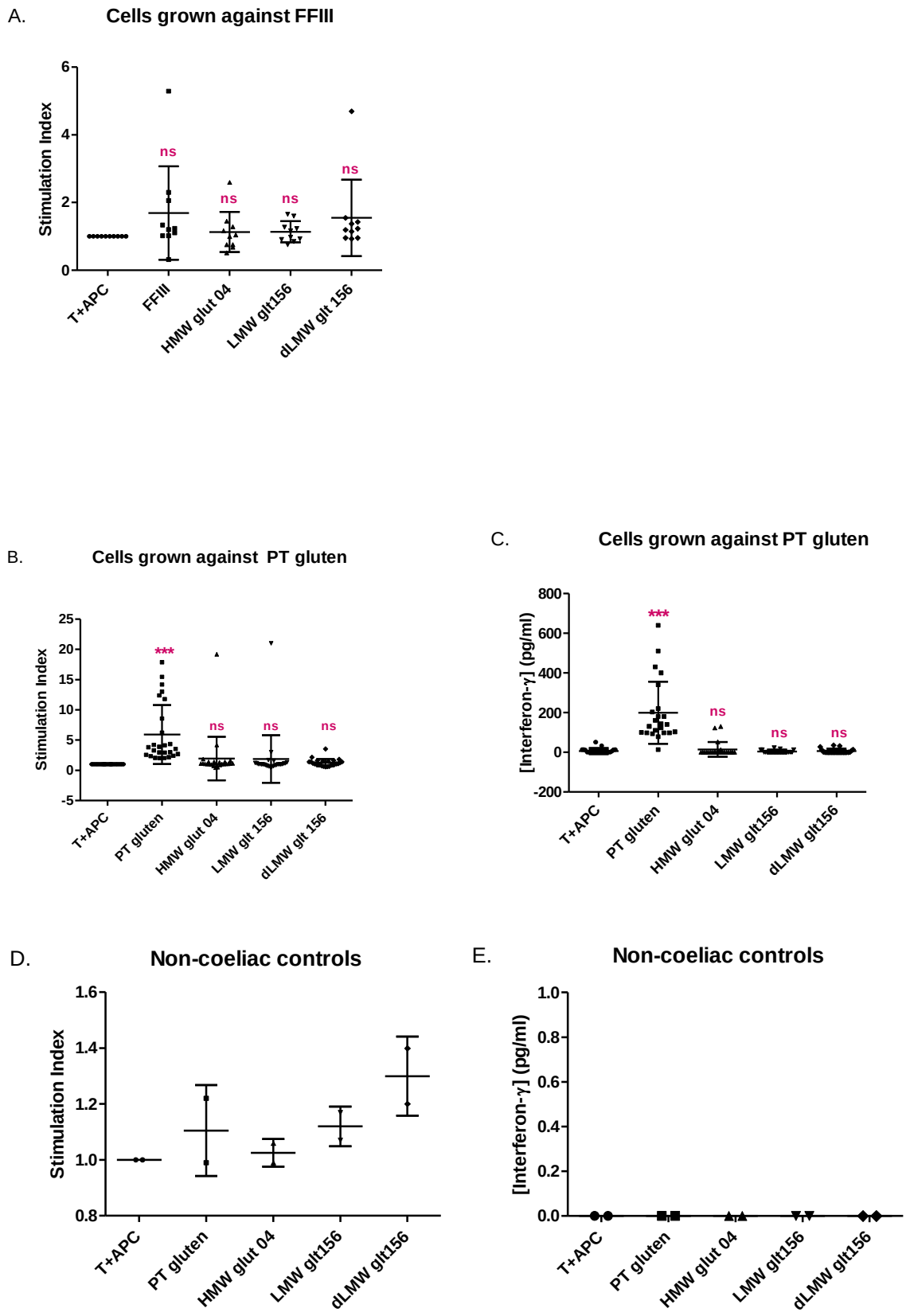


Figure legend explained overleaf.

This figure is a summary of all the values for each individual experiment. Each dot represents a value for an experiment, the bar indicates the mean with standard deviation as the tails. Stimulation index is calculated by dividing the mean counts per minute (CPM) for the T-cells plus antigen-presenting cells (APC) by the CPM for the T-cells plus APC alone after tritiated thymidine is added to the assay and incubated overnight (section 2.4.5). Interferon-γ concentrations were measured using ELISA from supernatants collected prior to adding the tritiated thymidine to the experiment (section 2.5.1). PT gluten=peptic-tryptic digested gluten, HMW glut 04=high molecular weight glutenin$_{04}$ 721-735, LMW glt156=low molecular weight glutenin glt$_{156}$ 44-59, dLMW glt 156=deamidated LMW glt$_{156}$ 44-59, FFIII=Frazer's Fraction III, ns=not significant, *** $p<0.0005$.

Figure 3.1A compares the mean stimulation index from proliferation assays of duodenal T-cells from coeliac patients grown *in vitro* against FFIII with the standard deviation shown as bars from tables 3.4-3.13. The mean stimulation index of T-cells with APC and no peptide is compared to the mean stimulation index of T-cells tested against positive control of FFIII and the candidate epitopes, HMW glut 04, LMW glt156 and LMT glt156 with standard deviation shown as the bars.

Figure 3.1B compares the mean stimulation index from proliferation assays of duodenal T-cells from coeliac patients grown *in vitro* against PT gluten from tables 3.15-3.42. The mean stimulation index of T-cells with APC and no peptide is compared to the mean stimulation index of T-cells tested against positive control of PT gluten and the candidate epitopes, HMW glut 04, LMW glt156 and LMT glt156 with the bars representing standard deviation.

Figure 3.1C compares the mean interferon-γ secretion from each test condition from proliferation assays of duodenal T-cells from coeliac patients grown *in vitro* against PT gluten from tables 3.15-3.42. The mean interferon-γ secretion of T-cells with APC and no peptide is compared to the mean interferon-γ secretion of T-cells tested against positive control of PT gluten and the candidate epitopes, HMW glut 04, LMW glt156 and LMT glt156 with the standard deviation represented by the bars.

Figure 3.1D compares the mean stimulation index from proliferation assays of duodenal T-cells from non-coeliac control patients grown *in vitro* against PT gluten from tables 3.44 and 3.45. The mean stimulation index of T-cells with APC and no peptide is compared to the mean stimulation index of T-cells tested against PT gluten and the candidate epitopes, HMW glut 04, LMW glt156 and LMT glt156 with the bars representing standard deviation.

Figure 3.1E compares the mean interferon-γ secretion from each test condition from proliferation assays of duodenal T-cells from non-coeliac control patients grown *in vitro* against PT gluten from tables 3.44 and 3.45. The mean interferon-γ secretion of T-cells with APC and no peptide is compared to the mean interferon-γ secretion of T-cells tested against PT gluten and the candidate epitopes, HMW glut 04, LMW glt156 and LMT glt156 with the standard deviation represented by the bars.

3.6 Discussion

At the start of this research, little was known on the immunostimulatory potential of glutenins and in particular, the disease-stimulating epitopes. The evidence available pointed to a region in the HMW protein (van de Wal 1999) found by a single HLA-DQ8 restricted T-cell clone in a child. Dewar *et al* (2006) demonstrated in 11/17 coeliac small intestinal T-cell lines that there was evidence of T-cell activation against a mixture of chemically purified high molecular weight glutenin. Villous atrophy developed when this mixture of chemically purified HMW glutenin protein (Dewar 2006) or recombinant Dy10 and Dx5 peptides (Ellis 2006) were instilled in the duodena of two coeliac patients demonstrating *in vivo* immunostimulation. There was less evidence for the immunostimulatory potential of LMW protein in adults with coeliac disease (Vader 2002a). During the course of this research, Tye-Din *et al* (2010) eloquently demonstrated that the HMW and LMW glutenin peptides tested on a large bank of peripheral blood lymphocytes taken from coeliac patients following a five-day oral gluten challenge failed to stimulate T-cells in the majority of HLA-DQ2 positive coeliac individuals. However, in a small minority of his patients, there was evidence of a marked T-cell response as assessed by enzyme-linked immunosorbent spot (ELISPOT). HMW $glut_{04}$ peptide is contained in W24, one of the peptides tested by Tye-Din whereas the LMW glt_{156} peptide used in this study failed to elicit a response in Tye-Din's screening phase and so was not tested further.

High molecular weight glutenin protein takes 68 hours to deamidate in a laboratory setting therefore HMW glut04 was tested in its native form rather than deamidated. Some LMW glutenins can be deamidated and so it was thought preferable to test both the native form of LMW glt156, as well as deamidated peptide.

The control patients were tested to ensure that the peptides were not immunostimulatory in non gluten-sensitive cell lines. More patients could have been tested; however, it was difficult to grow these cell lines without antigenic stimulation and it was difficult to obtain ethical approval for a large number of controls to be used.

Cell lines grown against FFIII did not yield gluten-sensitive cells in the majority of those tested. There was only one cell line (SD17) in whom FFII sensitive cells showed a weak response to HMW glut04. Interestingly, SD20, an HLA-DQ2/DQ8 heterozygous patient, had a positive stimulation index towards deamidated LMW glt156 despite the fact that the

T-cells were not FFIII sensitive cells. There was not enough information to say if this was potentially an HLA-DQ8 epitope due to lack of HLA-DQ8 positive patients.

All coeliac small intestinal T-cell lines grown against PT gluten demonstrated gluten sensitivity by positive stimulation index and at least a ten-fold rise in IFN-γ secretion, in the majority of those supernatants tested. However, only in a small minority of coeliac individuals tested was there any evidence of T-cell stimulation by HMW glut04, LMW glt156 or deamidated LMW glt156, that is in three, two and two out of twenty-eight individuals respectively. Interestingly, in the case of the one patient who had a positive stimulation index against LMW glt156 and deamidated LMW glt156 and for whom concentration of IFN-γ was known, there was less secretion of IFN-γ into the culture supernatant compared to that of PT gluten or HMW glut04.

These results from small intestinal T-cell lines of coeliac individuals, agree with the findings of Tye-Din's study (2010) from peripheral blood lymphocytes of coeliac individuals. In Tye-Din's study however, the LMW peptides were not immunostimulatory towards HLA-DQ2 peripheral blood coeliac T-cells and therefore they did not go on to further assessment in their manuscript. In the present study, the majority of T-cells from the small intestine of the coeliac individuals tested fail to be stimulated by both HMW glut04 and LMW glt156, whether or not it had been deamidated. There were not enough HLA-DQ8 individuals, homozygous or heterozygous, recruited to this study to evaluate the earlier effect seen in a FFIII cell line. It would be interesting to repeat this study in such individuals although, due to the paucity of such individuals this may not be feasible.

What this study does demonstrate is the heterogeneous response to different antigenic stimuli that adult coeliac patients have. Positive stimulation indices were seen in a small minority of patients. The group which underwent antigenic stimulation with PT gluten (SD38 and SD 73) seemed to respond to the glutenins early on, after a week of antigenic stimulation. This may be more representative of the mass T-cell gut action as the longer cells are in culture the more likely there is to be a clonal expansion of T-cell population which may not be naturally dominant.

The paediatric literature (van der Wal 1999, Vader 2002) used a small group of HLA DQ8 clones to test the immunostimulatory potential of both the HMW and LMW peptides tested in this study. In this study SD20 was the only HLA DQ2/8 heterozygote who demonstrated an immunostimulatory response towards the deamidated LMW glt156 peptide. The other

patients tested (SD17, SD 38 and SD73) were HLA DQ2 patients who demonstrated some response to the HMWglut04 peptide.

The best response to both the HMW glut04 peptide as well as the LMW glt156 peptide was an HLA DQ2 patient, SD17, who had undergone 2 rounds of FFIII antigenic restimulation. This result was unlikely to be due to bacterial contamination as the other wells did not respond in the similar way; if bacteria were to come in contact with a plate it is highly unlikely that only 6 wells would be affected as bacteria are highly contagious in cell culture. This was a young patient with a reasonably new diagnosis of coeliac disease made 4 months prior to recruitment. It may be that earlier in the disease more epitopes are able to elicit a T-cell response. This theory is supported by Vader's work (2002a) that demonstrated that the paediatric clonal T-cell population responded differently to antigenic stimulation when compared to a smaller group of adult HLA DQ2 clonal T-cell population. This study lends more weight to the argument of epitope-spreading in children with epitope-focussing in adults. In children, the immune response is capable of reacting to any number of epitopes but that T-cell response will eventually focus on those peptides that combine strong HLA DQ binding with potent T-cell activity to reduce the number of epitopes to which adults will mount a response. By testing paediatric epitopes HMW glut04 and LMW glt156 in an adult population these have been shown not to be major T-cell epitopes. However, perhaps if tested in HLA DQ8 adults or in a group of recently diagnosed coeliac population these epitopes may be more immunogenic than first thought.

Chapter 4: The role of glutenins in innate immunity

4.1 Introduction and Aims

Initial reports of organ culture of coeliac disease-affected small intestinal mucosa came from Trier and Browning in 1970. They showed that untreated coeliac mucosa, after only 24 hours of organ culture in a gluten-free environment, reverted towards normal; the enterocytes became more columnar and less vacuolated. By ^{3}H thymidine incorporation into crypt cells, they also demonstrated that untreated coeliac mucosa has an enhanced epithelial cell proliferation compared with normal mucosa, and that this proliferation returns to normal in patients treated with a gluten-free diet. This demonstrated that it was possible to study coeliac mucosa *in vitro* and differentiate it from normal mucosa.

Falchuk *et al* (1974) published their first report of a series of experiments aimed at studying the pathogenesis of the mucosal abnormality in coeliac disease. They demonstrated that the mucosa of coeliac patients behaved differently in the presence of Frazer's Fraction III by measuring changes in alkaline phosphatise activity, and found that these changes were specific for gluten. The inhibition of the rise in alkaline phosphatise activity with gluten was not present when casein was in the medium. However, there was some disagreement about the biochemical changes initially reported by Falchuk, with others not finding them so reproducible (Hauri 1978, Howdle 1981a, Fluge 1982).

Howdle *et al* (1981b) modified this technique to use morphometric assessment as a reliable and reproducible means of studying coeliac disease. They measured the height of enterocytes (ECH) on small intestinal biopsies pre- and post-culture as well as measuring intra-epithelial lymphocytes (IELs) per 100 enterocytes and the crypt depth:villous height ratio. They were able to show that the reduction in ECH was specific for gluten and for coeliac mucosa. This modified model has been used to test the toxicity of various gluten sub-fractions (Ciclitira 1984, de Ritis 1988, Przemioslo 1995, Shidrawi 1995, Maiuri 1996, Biagi 1999, Beckett 1999, Martucci 2003, Mazzarella 2003, Maiuri 2003).

Maiuri *et al* (2003) demonstrated that A-gliadin p31-43, a gluten peptide, could damage small intestinal biopsies in organ culture, via interleukin-15, despite not stimulating gluten-sensitive T-cells (see section 1.10). It is thought that IL-15 activates IELs (Di Sabatino

2006, Mention 2003, Bergamashi 2008) and therefore can be seen as a link between the innate and adaptive immune response. Some gluten peptides can induce mucosal damage in coeliac disease through direct activation of the innate immune system. IL-15 is a protein mainly expressed by fibroblasts, epithelial cells, monocytes and dendritic cells. It is essential for the development of natural killer and T-cells (see section 1.4.1) and has a potent anti-apoptotic effect on these cells (Fehniger 2001, Budagian 2006).

Refractory coeliac disease (RCD) is a rare complication of coeliac disease, see section 1.7.1, where interleukin-15 seems to play an important role in disease pathogenesis. Mention *et al* (2003) found that IL-15 was overexpressed by enterocytes in both coeliac disease and RCD. More recently, Malamut *et al* (2010) found that when IL-15 was blocked by antibodies, the massive accumulation of intra-epithelial lymphocytes normally seen in RCD was reduced. The pathophysiology of refractory coeliac disease is poorly understood. This is due in part to the scarcity of patients. Even less is known about the effect of glutenin peptides in RCD. St Thomas Hospital is a tertiary referral centre for refractory coeliac patients for the South East of Britain and from further afield in the UK. It was hoped that this patient group could be used in the assessment of the role of glutenins in the innate immune response. The results from refractory coeliac disease duodenal biopsies have never been published in organ culture systems. The T-cells from the small intestine of RCD patients are known to be difficult to grow *in vitro.*This is due to their lack of antigenic stimulation by gluten as well as the immunosuppression treatment regimens, which patients are often on to help control their disease, to reduce the lymphocyte count.

Results from T-cell proliferation studies (see Chapter 3) suggested that HMW $glut_{04}$ and LMW glt_{156} were not major T-cell epitopes. They were unable to be tested in RCD patients as the T-cells were not able to be grown *in vitro*. These two glutenin peptides HMW $glut_{04}$ and LMW glt_{156}, have not previously been used in small intestinal organ culture systems to assess their activation of the innate immune system.

The aim of this study was to assess the innate immune system response towards two glutenin candidate epitopes, HMW $glut_{04}$ and LMW glt_{156}, in both coeliac disease and type 2 refractory coeliac disease. Enterocyte cell height (ECH) was measured in formalin-fixed paraffin–embedded duodenal biopsy sections stained with haematoxylin and eosin and interleukin-15 secretion into the culture supernatants was assessed by high sensitivity ELISA. The null hypothesis in these experiments is that there is no clinically significant

histological change in the biopsies after overnight incubation with candidate epitopes, HMW $glut_{04}$ and LMW glt_{156} glutenin peptides.

4.2 Methods

4.2.1 Small intestinal biopsy organ culture

The methods used in this chapter were based on those reported by Przemioslo (1995) where biopsy viabiltity was assessed after delay in the organ culture system and length of time in the organ culture system. Pairs of small intestinal biopsies were set up, touching each other, in separate organ culture dishes containing either medium-only, ovalbumin as a negative control, PT gluten as a positive control, HMW $glut_{04}$ or LMW glt_{156} as described in section 2.3. Proteins (PT gluten and ovalbumin) were used at 1mg/ml concentration whereas the peptides (HMW $glut_{04}$ and LMW glt_{156}) were used at 200µg/ml.

After overnight incubation the supernatants were collected, the biopsies were weighed, and fixed in formal saline as per section 2.6.1. After 48 hours in formal saline the biopsies were embedded in paraffin, see section 2.6.2.

Secretory interleukin-15 in the organ culture supernatant was measured by ELISA, as per section 2.6.2, on a single day.

4.2.2 Processing of formalin-fixed, paraffin-embedded small intestinal biopsies

Tissue blocks were cut at 4µm thickness and baked on Super Frost sides at 60°C overnight (kindly done by Prof Novello's team at University College Hospital, London). They were then de-waxed, stained with haematoxylin and eosin (H+E), to measure enterocyte cell height, or interleukin-15 immunohistochemistry as per sections 2.6.2, 2.6.3, 2.6.4, 2.6.5 and 2.6.6.

4.2.3 Microscopic examination of the slides

The H+E stained slides were examined under blinded conditions using low power (x100) to identify the anatomy. These blinded conditions were unblended at the end of the measurement of the entire patient cohort.

Where visible, three villi were selected in order to measure the enterocyte cell height ECH). Enterocytes were counted at the middle of the villi where there is the least amount of ECH variation. At each villi, 30 continuous enterocytes were measured at high power (x400)

using a graticule, giving a total of 90 ECH measurements for each section. This graticule required a coefficient of x1.92 correction to the measurements obtained.

Where the biopsies were a Marsh 3, villi were not able to be identified however, the enterocyte layer was clear and the measurements were taken from the middle of the section. Measurements were not taken from any villi or epithelial layer at the edge of the section due to any artefact in the tissue handling or processing, as is standard among histopathologists. In damaged biopsies, the enterocyte layer was occasionally detached from the epithelium and this was stated in the results.

4.2.4 Statistics

In order to decide on relevant statistical tests the mean and median enterocyte cell height of each sample group were compared, see table 4.1 and 4.2. These tables include the Marsh grading (Oberhuber 1999) given by the gastrointestinal pathologist at St Thomas Hospital reporting the biopsy sent as part of routine investigations in these patients. They demonstrate that there is no clinically significant difference between the mean and median for each patient in the coeliac as well as type 2 refractory coeliac groups therefore normal distribution was assumed.

GraphPad Prism 5.0 for Windows was used in the statistical analysis in this chapter. A one-way repeated measurement ANOVA with Dunnett's multiple comparison test was used to compare the median of medium-only against all other groups within the same patient.

Table 4.1 Comparison of mean and median enterocyte cell height (microns) in coeliac population

Patient (Marsh)	medium		ovalbumin		PT gluten		HMW $glut_{04}$		LMW glt_{156}	
	mean (SEM)	median	mean (SEM)	median	mean (SEM)	median	mean (SEM)	median	mean (SEM)	median
SM2 (3b)	16.5 (0.9)	16.3	17.3 (0.5)	17.3	13.1 (1.0)	12.1	14.9 (1.0)	14.9	11.2 (0.5)	10.9
SM4 (SD20) (3a)	26.4 (0.7)	26.8	36.2 (0.7)	37.1	15.4 (0.5)	15.4	16.8 (0.5)	17.3	13.2 (0.5)	12.8
SM5 (1-2)	28.9 (0.7)	28.8	28.1 (0.6)	27.2	10.8 (0.5)	10.2	14.6 (0.4)	14.4	25.1 (0.8)	26.2
SM6 (3a)	39.9 (0.8)	39.4	49.3 (0.9)	49.9	17.4 (0.4)	17.9	29.9 (0.9)	29.4	15.7 (0.5)	15.4
SM9 (1)	32.5 (0.9)	32.6	47.5 (0.9)	46.7	23.5 (1.0)	23.7	17.9 (0.9)	17.9	24.7 (0.9)	25.0
SM10 (SD50) (0)	40 (1.1)	38.0	35 (1.0)	35.2	24.0 (0.9)	25.0	27.8 (0.7)	27.5	28.5 (0.6)	28.9
SM12 (0)	54.9 (1.0)	54.7	45.9 (1.0)	46.1	43.0 (1.4)	44.4	22.1 (1.4)	21.8	23.3 (1.1)	24.3
SM15 (SD63) (3a-b)	17.9 (0.6)	17.6	14.8 (0.6)	15.0	21.2 (1.2)	20.2	32.0 (1.5)	31.4	36.9 (1.6)	35.8
SM17 (0)	21.9 (1.1)	22.1	29.7 (1.4)	28.8	25.5 (1.1)	27.8	17.9 (0.9)	17.3	Biopsy destroyed	

Paired biopsies were left overnight in an organ culture system under five different conditions: medium-only, ovalbumin only as a negative peptide, peptic-tryptic digest of gluten (PT gluten) as a positive control, high molecular weight $glutenin_{04}$ 721-735 (HMW glut04) and low molecular weight glutenin glt_{156} 44-59 (LMW glt156). They were placed in formal saline for 48 hours before embedding in paraffin. Four micron sections were stained with haematoxylin and eosin and enterocyte cell height was measured at mid villus in 30 continuous enterocytes in three villi.

Table 4.2 Comparison of mean and median enterocyte cell height (microns) in type 2 refractory coeliac population

Patent (Marsh)	Medium		Ovalbumin		PT gluten		HMW $glut_{04}$		LMW glt_{156}	
	mean (SEM)	**median**	**mean (SEM)**	**median**	**mean (SEM)**	**median**	**mean (SEM)**	**median**	**mean (SEM)**	**median**
SM3 (3b)	23.8 (1.1)	23.7	17.3 (0.7)	17.3	Biopsy destroyed		Biopsy destroyed		9.5 (0.5)	9.6
SM7 (0)	28.6 (0.6)	27.5	29.1 (0.8)	28.8	12.5 (0.7)	11.5	12.2 (0.5)	11.5	22.3 (0.8)	22.4
SM8 (3b)	35.0 (0.9)	34.2	38.3 (1.0)	39.4	28.9 ((1.1)	28.2	26.8 (0.9)	26.2	20.4 (0.7)	20.5
SM13 (0)	45.0 (1.0)	45.8	41.0 (1.1)	42.2	17.8 (0.7)	17.9	21.7 (0.8)	21.1	31.0 (0.9)	31.4
SM16 (0)	30.7 (1.1)	30.8	33.4 (0.9)	33.9	27.7 (1.0)	29.1	26.1 (1.2)	25.9	18.2 (1.0)	18.6

Paired biopsies were left overnight in an organ culture system under five different conditions: medium-only, ovalbumin only as a negative peptide, peptic-tryptic digest of gluten (PT gluten) as a positive control, high molecular weight $glutenin_{04}$ 721-735 (HMW glut04) and low molecular weight glutenin glt_{156} 44-59 (LMW glt156). They were placed in formal saline for 48 hours before embedding in paraffin. Four micron sections were stained with haematoxylin and eosin and enterocyte cell height was measured at mid villus in 30 continuous enterocytes in three villi.

4.3 Peptides

The rationale for choosing the peptides in this study has already been explained in section 1.12. The following peptides were made commercially by GenScript (New Jersey, USA) with the minimum stimulating T-cell epitope in bold and the underlined glutamine residues a target for deamidation to glutamic acid:

Amino acids 721-735 of HMW glutenin, HMW glutenin 04 (HMW glut04)

QGQ**QGYYPTSPQ**QSG

Amino acids 44-59 of LMW glutenin LMW glutenin 156 (LMW glt156)

PPFSQQQQSPFSQQQQ

As deamidation of peptides has been shown to occur by endogenous tissue transglutaminase in the mucosal tissue in culture (Molberg 2001), LMW glutenin glt156 was not deamidated prior to addition in the organ culture system.

4.4 Patients used in small intestinal biopsy organ culture studies

Three different patient groups were used in this study. The first group consisted of nine coeliac patients who were undergoing endoscopy as part of their routine clinical work up who consented to the study. The demographic details of these patients are shown in table 4.3. Interestingly, three of the patients, SM4, SM10 and SM15, also took part in the T-cell experiments in Chapter 3, known as SD20, SD50 and SD63. These are highlighted on the table with their Chapter 3 labels in brackets.

Most of these patients had been diagnosed with coeliac disease and established on a gluten-free diet for a number of years. The length of time patients had coeliac disease was taken as the time from their first diagnostic small intestinal biopsy. There was only one HLA DQ8 positive patient in this cohort which is in keeping with prevalence of HLA DQ8 in the coeliac population. The ages span from 24 to 79 with the majority of patients agreeing to take part in the study being female.

Table 4.3 Coeliac disease patients in small intestinal organ culture studies

Patients	Age (years)	Sex	HLA status	Length of time with coeliac disease	Other diagnoses
SM2	39	F	DQ2	New diagnosis	Protein C deficiency, OA, varicose veins
SM4 (SD20)	24	F	DQ8	3 years	nil
SM5	79	M	DQ2	14 years	Lung cancer, colectomy for multiple polyps
SM6	20	F	DQ2	4 years	PCOS, asthma
SM9	32	F	DQ2	11 years	Previous *Helicobacter pylori* gastritis
SM10 (SD50)	60	F	DQ2	12 years	Psoriasis, meningioma resected
SM12	61	M	DQ2	4 years	nil
SM15 (SD63)	48	F	DQ2	4 years	SLE, recurrent GU
SM17	37	F	DQ2	2 years	nil

Number in bracket relates to the patient number in Chapter 3

As St Thomas Hospital is a referral centre for refractory coeliac disease, there was wealth of these patients available for assessment with no previously published organ culture studies. The second group in this study were therefore five patients who had a diagnosis of type 2 refractory coeliac disease and were undergoing endoscopic assessment every four months as part of their disease evaluation who consented to the study. The demographics for these patients are demonstrated in table 4.4. The length of time patients had coeliac disease was taken as the time from their first diagnostic small intestinal biopsy. The length of time patients had a diagnosis of type 2 RCD was taken as the time from first histological biopsy demonstrating Marsh 3 changes (section 1.5.2) intraepithelial lymphocyte T-cell receptor monoclonality demonstrated while on a gluten-free diet (section 1.6.1) and no other cause of villous atrophy identified.

As with RCD, they were of an older age range 53-75 and were more evenly matched for sex than the coeliac patients in table 4.1. There was only one HLA DQ8 patient in this study however, this is a little higher than would be expected by chance.

Table 4.4 Type 2 refractory coeliac disease patients in small intestinal organ culture studies

Patients	**Age (years)**	**Sex**	**HLA status**	**Length of time with coeliac disease**	**Other diagnoses**
SM3	69	F	DQ2	CD 3 years RCD 1 year	nil
SM7	53	F	DQ2	CD 24 years RCD 6 years	nil
SM8	67	M	DQ8	CD 7 years RCD 3 years	Depression, alcohol excess
SM13	64	M	DQ2	CD 14 years RCD 13 years	EATL, cardiomyopathy, stem cell transplant
SM16	75	F	DQ2.5	CD 8 years RCD 2 years	RA, GORD

CD coeliac disease, RCD type 2 refractory coeliac disease

The third group in this study was a non-coeliac control group of only two patients due to our available ethics. They were undergoing routine endoscopy as part of their clinical investigation and consented to the study. The purpose of this small group was to try to demonstrate that the candidate glutenin epitopes did not produce a toxic response in the small intestinal biopsies.

Both patients had been screened with an IgA and IgG tissue transglutaminase antibody as well as IgA anti-endomysial antibody to exclude coeliac disease. The formal histology from the GI pathologists at St Thomas Hospital did not show any evidence of coeliac disease.

The patient demographics in this group are shown in table 4.5. They are young patients with only one HLA DQ status available which was neither HLA DQ2 nor 8 positive. Both patients were young and both sexes were represented.

Table 4.5 Non-coeliac disease patients in small intestinal organ culture studies

Patients	Age (years)	Sex	HLA status	Diagnoses
SM11	48	F	NA	Depression, vertigo, GORD, stillbirth
SM14	30	M	–ve	IBS

NA not available, -ve HLA DQ2 and 8 negative

4.5 Results

Appendix V contains photographs of some H+E slides analysed in the sections below.

In organ culture biopsies it is not unusual to see “debris” on the luminal surface of the biopsy where dead enterocytes and inflammatory cells have sloughed off the biopsy. There is often a slight difference in histologic appearances in organ culture biopsies as there is often evidence of autolysis occurring. This is due to the delay in fixing the tissue. Whereas biopsies taken for histology in endoscopy are put immediately into formal saline, there is a delay of 18 hours in the organ culture sytem. Tables 4.6, 4.7 and 4.8 also contain the GI pathogists' assessment of the Marsh grading from the biopsies sent at the time of endoscopy which agreed with the organ culture system assessment of the medium-only biopsy specimens.

Box plots are used in Figures 4.1, 4.2 and 4.3 to depict the spread of data from the repeated measurements of enterocyte cell height. The sample minimum and maximum values are whiskers extending from the box, the upper and lower quartiles make the box and median is shown as a line in the box.

4.5.1 Results from the coeliac patients

Every patient had a pair of biopsies in each experimental condition in the organ culture system; medium alone to act as the reference; ovalbumin, a neutral protein to act as negative control; peptic-tryptic digest of gluten as the positive control; the candidate glutenin peptides HMW glut04 and LMW glt156.

Figure 4.1A-I demonstrate the results of enterocyte cell height (ECH) for each coeliac patient. There is often a significant difference seen between the medium-only group and the ovalbumin group which can be accounted for in biopsy size. The smaller biopsies were used in medium-only rather than the other experimental conditions. This explains why the ECH is often better in the ovalbumin group, although histopathologists consider a normal enterocyte height to be between 30 and 40 microns. Figure 4.1A is of a newly diagnosed coeliac patient hence the smaller ECH across all experimental conditions.

There is a clear significant reduction in ECH in the PT gluten biopsies as well as both candidate glutenin epitopes seen across all the coeliac patients. In Figures 4.1B, F and H there is also T cell data from Chapter 3 demonstrating that in these three patients there was no T cell stimulation with the HMW glut04, LMW glt156 and deamidated LMW glt156

peptides. There is a significant reduction in the ECH in each of these three patients with both the HMW glut04 peptide and the LMW glt156 peptide.

Unfortunately in Figure 4.1I patient SM17 LMW glt 156 biopsy was destroyed by the incubation and therefore ECH could not be determined. This was not as a result of bacterial contamination as the medium would not have remained the same colour, nor was it likely due to technique as the other biopsies would have also been destroyed. This further reinforces the idea that *in vitro* these glutenin peptides are certainly harmful to coeliac mucosa.

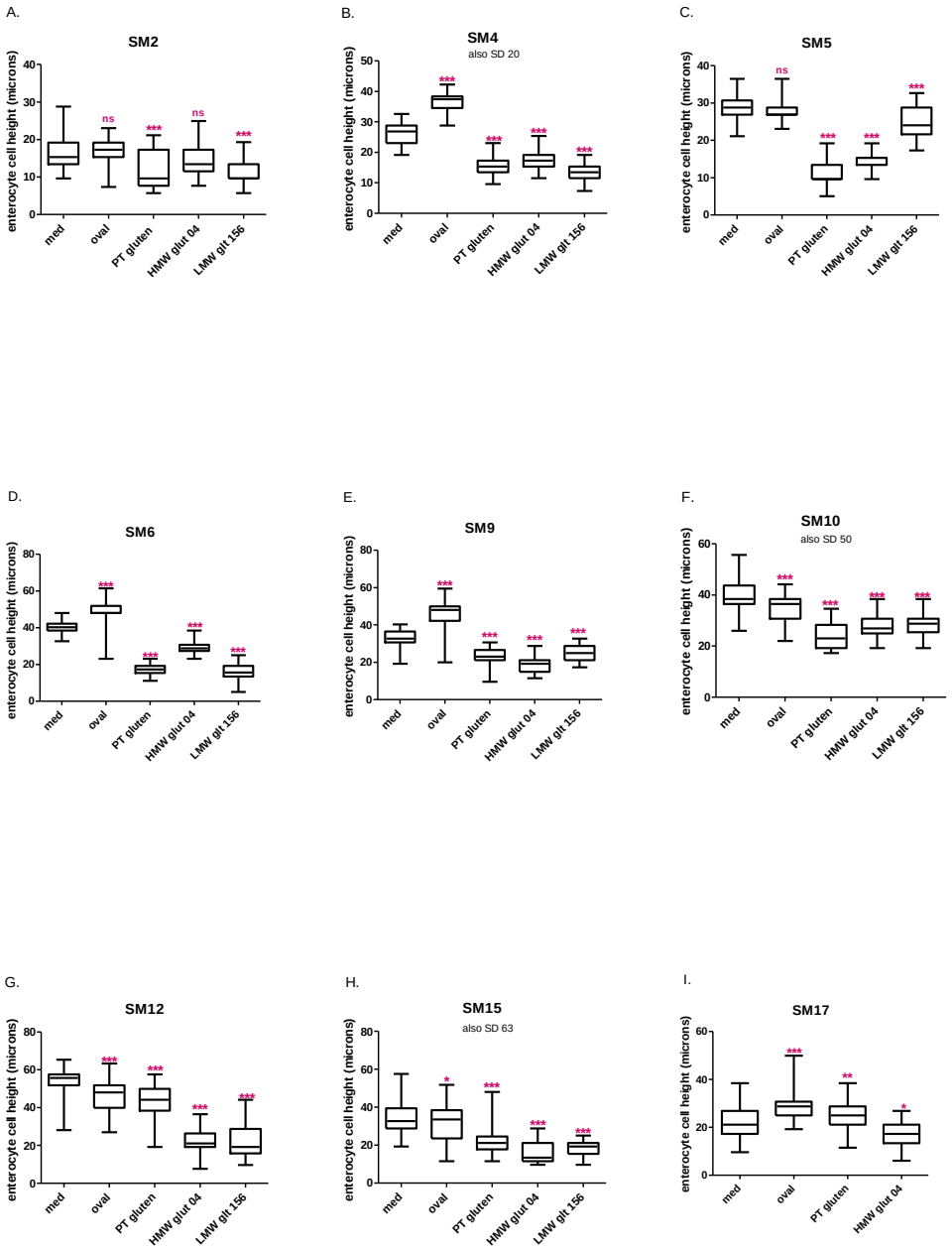
Figure 4.2 demonstrates the combined results of all the coeliac patients. Overall it demonstrates, as expected, the PT gluten ECH is significantly reduced but that both the HMW glut04 and LMW glt156 ECH are also reduced significantly. This suggests that these peptides do trigger an immune response in the mucosa of coeliac patients.

These biopsies were then stained for interleukin-15 (IL-15) on different sections using immunohistochemistry in order to see if this response involved IL-15. Unfortunately this staining was not successful after review by the histopathologist. Secretory IL-15 was measured in the organ culture medium which had been frozen at -20°C to enable all samples to be tested on the same day. The results are demonstrated in table 4.6 with the secretory IL-15 data expressed as picograms per milligram of tissue. The Marsh grading given by the GI histopathologists at St Thomas Hospital is given in brackets below the patient details.

There is little IL-15 secreted into the organ culture medium as measured by high sensitivity ELISA.

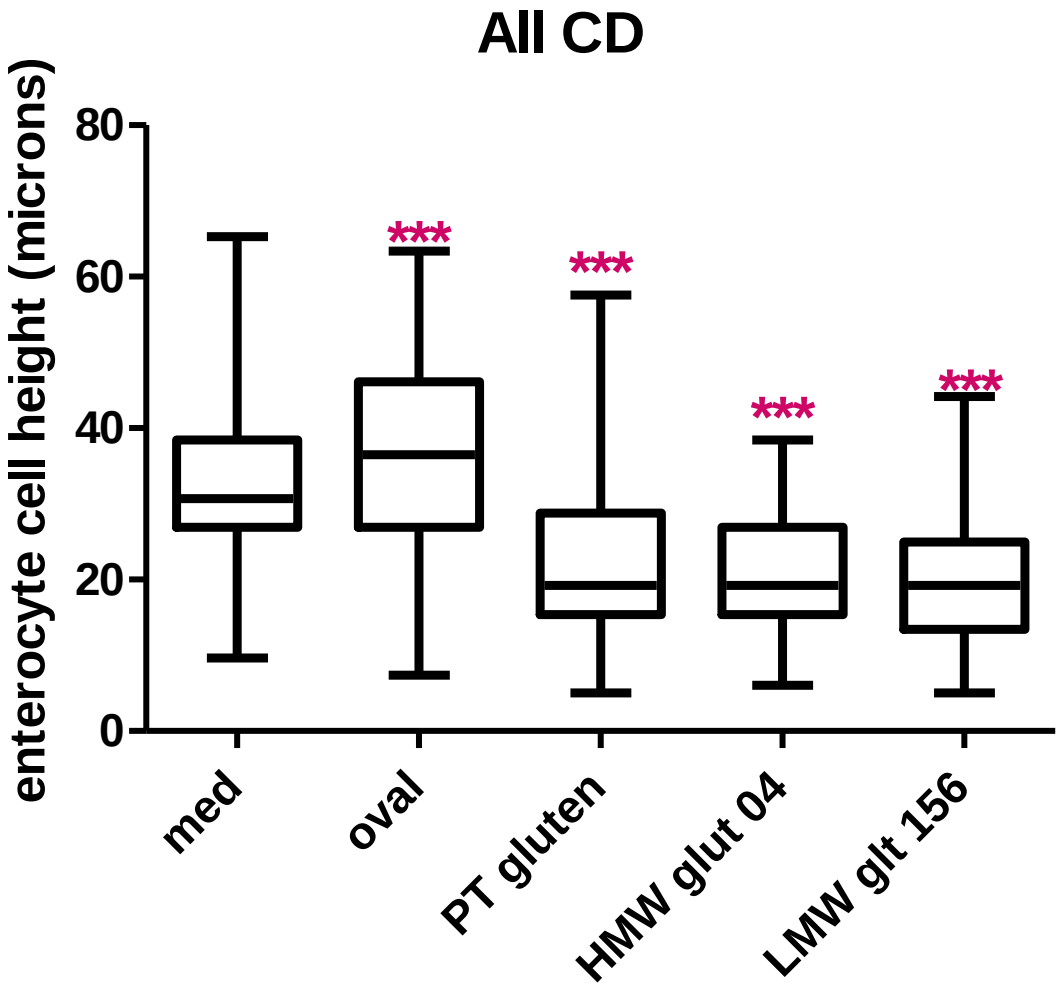
There was only one patient who was HLA DQ8, SM4, and this patient did not appear to behave any differently to the other patients in this study.

Figure 4.1 Individual box plots for coeliac patients



Results of overnight incubation of a pair of coeliac duodenal biopsies in organ culture system with medium-only (med), ovalbumin (oval), a peptic-tryptic digest of gluten (PT gluten) and the candidate glutenin epitopes HMW glut04 (high molecular weight glutenin$_{04}$ 721-735) and LMW glt156 (low molecular weight glutenin glt$_{156}$ 44-59). The biopsy morphology was preserved in formal saline for 48 hours and embedded in paraffin wax, after which they were cut 4μm thick, dewaxed and stained with haematoxylin and eosin. At x400 magnification the enterocyte cell height from mid-villus was counted for 30 continuous enterocytes in three different villi. The median, interquartile range and maximum and minimum values are represented in the graphs above. Each graph, A-I, is a different patient. Median values are compared to medium-only to see if there is any difference. ns=not significant, * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

Figure 4.2 Box plots for all coeliac patients



Combined results for each coeliac patient from Figure 4.1 enterocyte cell height measurements after overnight incubation of a pair of coeliac duodenal biopsies in organ culture system with medium-only (med), ovalbumin (oval), a peptic-tryptic digest of gluten (PT gluten) and the candidate glutenin epitopes HMW glut04 (high molecular weight glutenin$_{04}$ 721-735) and LMW glt156 (low molecular weight glutenin glt$_{156}$ 44-59). The biopsy morphology was preserved in formal saline for 48 hours and embedded in paraffin wax, after which they were cut 4µm thick, dewaxed and stained with haematoxylin and eosin. At x400 magnification the enterocyte cell height from mid-villus was counted for 30 continuous enterocytes in three different villi. The median, interquartile range and maximum and minimum values are represented in the graphs above. Median values are compared to medium-only to see if there is any difference. ns=not significant, * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

Table 4.6 Interleukin-15: ELISA quantification of secretory interleukin-15 results in coeliac patients

Patient (Marsh)	Medium ELISA (pg/mg)	Ovalbumin ELISA (pg/mg)	PT gluten ELISA (pg/mg)	HMW $glut_{04}$ ELISA (pg/mg)	LMW glt_{156} ELISA (pg/mg)
SM2 (3b)	0.00	0.00	0.00	0.00	0.02
SM4 (SD20) (3a)	0.02	0.00	0.00	0.04	0.06
SM5 (1-2)	0.01	0.00	0.02	3.54	0.06
SM6 (3a)	0.04	0.03	0.08	0.05	0.06
SM9 (1)	0.03	0.03	0.11	0.01	0.01
SM10 (SD50) (0)	0.03	0.07	0.11	0.03	0.03
SM12 (0)	0.04	0.01	0.06	0.05	0.02
SM15 (SD63) (3a-b)	0.01	0.01	0.02	0.03	0.02

Paired biopsies were left overnight in an organ culture system under five different conditions: medium-only, ovalbumin only as a negative peptide, peptic-tryptic digest of gluten (PT gluten) as a positive control, high molecular weight $glutenin_{04}$ 721-735 (HMW glut04) and low molecular weight glutenin glt_{156} 44-59 (LMW glt156). Secretory IL-15 was measured with a commercial high sensitivity ELISA kit from the culture supernatants.

4.5.2 Results from the type 2 refractory coeliac disease patients

Every patient had a pair of biopsies in each experimental condition in the organ culture system; medium alone to act as the reference; ovalbumin, a neutral protein to act as negative control; peptic-tryptic digest of gluten as the positive control; the candidate glutenin peptides HMW glut04 and LMW glt156.

Refractory coeliac disease biopsy specimens shown in Figure 4.3A-E demonstrate a similar trend as the coeliac patients in Figure 4.1. There is a difference between the ovalbumin group and the medium-only group which can be accounted for by biopsy size: the smaller biopsies being used in the medium-only group. They all show decreased ECH in the PT gluten group as well as both HMW glut04 and LMW glt156 groups. Unfortunately the biopsy specimens for SM3 in PT gluten and HMW glt04 were destroyed and therefore unable to measure ECH.

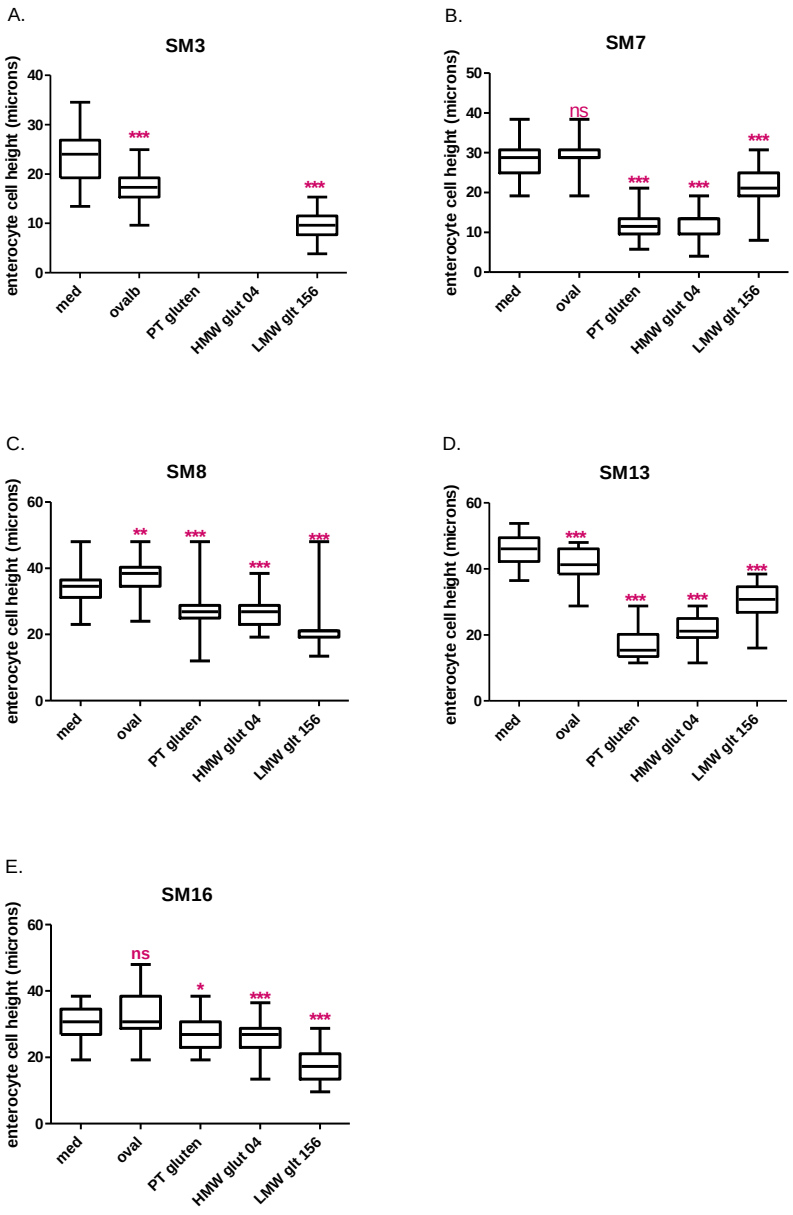
Figure 4.4 shows the combined ECH for each patient under each experimental condition. It summarises that there is an overall significant difference between the ECH in the medium-only group and the ECH in the PT gluten, HMW glut04 and LMW glt156 groups.

Both these figures demonstrate that there is a toxic process happening *in vitro* in the mucosa of type 2 refractory coeliac disease despite their treatment with azathioprine (2.5mg/kg) and 20mg prednisolone.

Unfortunately, the IL-15 immunohistochemistry was reviewed by histopathologist, Prof Novelli, who suggested it be removed from this study. Secretory IL-15 was measured by high sensitivity ELISA from the biopsy culture supernatants, the results expressed as picograms per milligram of tissue. The results of these experiments are shown in table 4.7 demonstrating there was little IL-15 secreted into the culture supernatants.

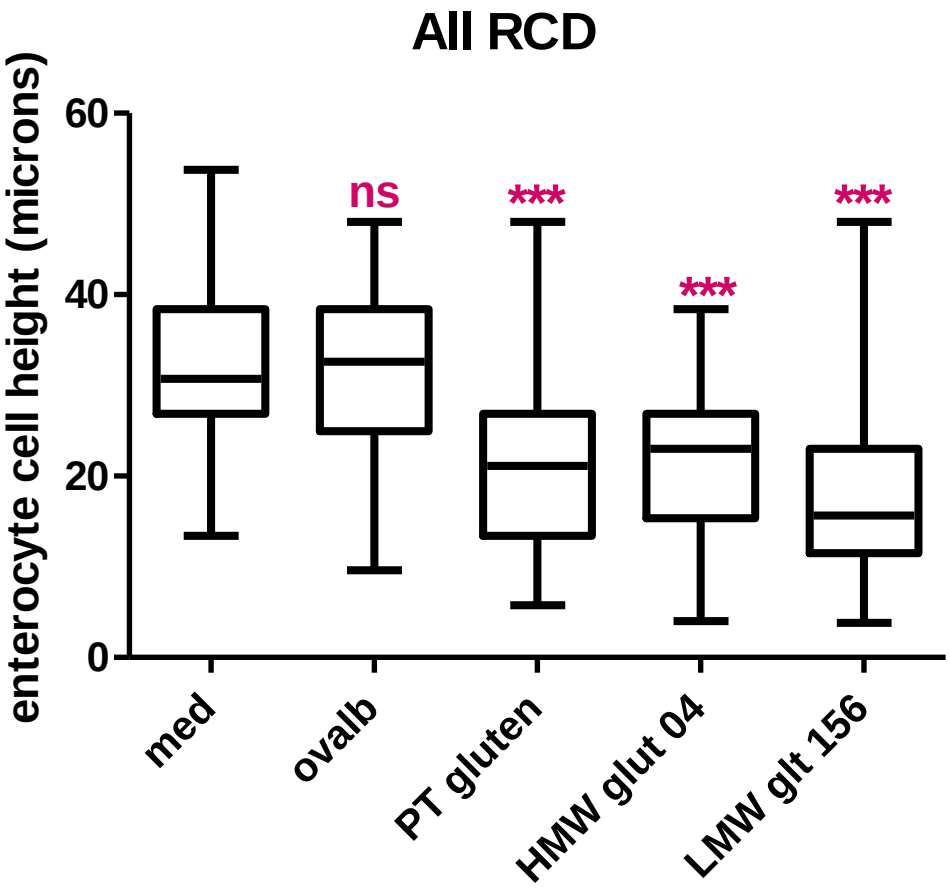
There was only one patient who was HLA DQ8, SM8, and this patient did not appear to behave any differently to the other patients in this study.

Figure 4.3 Individual box plots for type 2 refractory coeliac patients



Enterocyte cell height measurements after overnight incubation of a pair of type 2 refractory coeliac duodenal biopsies in organ culture system with medium-only (med), ovalbumin (oval), a peptic-tryptic digest of gluten (PT gluten) and the candidate glutenin epitopes HMW glut04 (high molecular weight glutenin$_{04}$ 721-735) and LMW glt156 (low molecular weight glutenin glt$_{156}$ 44-59). The biopsy morphology was preserved in formal saline for 48 hours and embedded in paraffin wax, after which they were cut 4µm thick, dewaxed and stained with haematoxylin and eosin. At x400 magnification the enterocyte cell height from mid-villus was counted for 30 continuous enterocytes in three different villi. The median, interquartile range and maximum and minimum values are represented in the graphs above. Each graph, A-E, is a different patient. Median values are compared to medium-only to see if there is any difference. ns=not significant, * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

Figure 4.4 Box plots for all type 2 refractory coeliac disease



Combined results of enterocyte cell height measurements for each type 2 refractory coeliac patient from Figure 4.3 after overnight incubation of a pair of coeliac duodenal biopsies in organ culture system with medium-only (med), ovalbumin (oval), a peptic-tryptic digest of gluten (PT gluten) and the candidate glutenin epitopes HMW glut04 (high molecular weight glutenin$_{04}$ 721-735) and LMW glt156 (low molecular weight glutenin glt$_{156}$ 44-59). The biopsy morphology was preserved in formal saline for 48 hours and embedded in paraffin wax, after which they were cut 4µm thick, dewaxed and stained with haematoxylin and eosin. At x400 magnification the enterocyte cell height from mid-villus was counted for 30 continuous enterocytes in three different villi. The median, interquartile range and maximum and minimum values are represented in the graphs above. Median values are compared to medium-only to see if there is any difference. ns=not significant, * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

Table 4.7 Interleukin-15: ELISA quantification of secretory interleukin-15 results in type 2 refractory coeliac patients

Patient (Marsh)	**Medium ELISA (pg/mg)**	**Ovalbumin ELISA (pg/mg)**	**PT gluten ELISA (pg/mg)**	**HMW $glut_{04}$ ELISA (pg/mg)**	**LMW glt_{156} ELISA (pg/mg)**
SM3 (3b)	0.00	0.02	0.09	0.01	0.03
SM7 (0)	0.01	0.07	0.10	0.63	0.3
SM8 (3b)	0.00	0.03	0.01	0.63	0.3
SM13 (0)	0.06	0.03	0.03	0.05	0.03
SM16 (0)	0.06	0.06	0.06	0.06	0.02

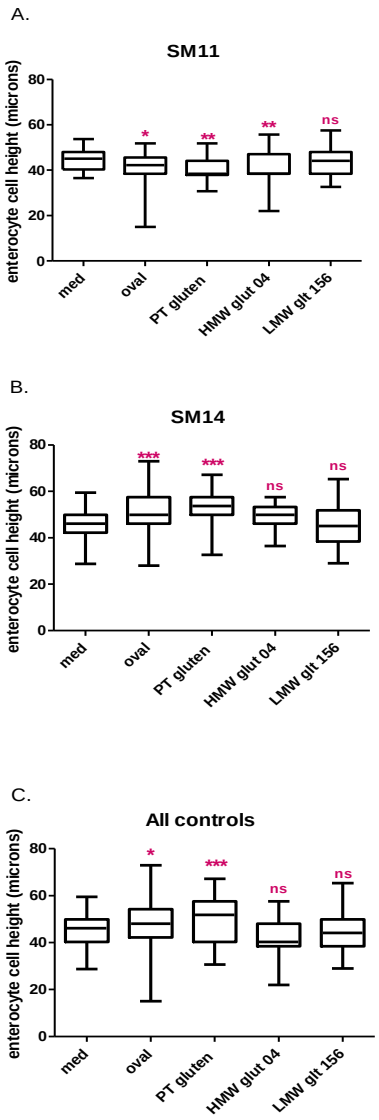
Paired biopsies were left overnight in an organ culture system under five different conditions: medium-only, ovalbumin only as a negative peptide, peptic-tryptic digest of gluten (PT gluten) as a positive control, high molecular weight $glutenin_{04}$ 721-735 (HMW glut04) and low molecular weight glutenin glt_{156} 44-59 (LMW glt156). Secretory IL-15 was measured with a commercial high sensitivity ELISA kit from the culture supernatants.

4.5.3 Results from the non-coeliac patients

Figure 4.5A and B demonstrate that the ECH is increased after overnight incubation with PT gluten or the candidate peptides, HMW glut04 and LMW glt156. In Figure 4.5C, where results from both patients are combined, there is no effect on the ECH with either of the candidate peptides. There is an amelioration of ECH in the biopsies incubated with PT gluten.

In table 4.8, when these biopsies were assessed for IL-15 production there is again little secretion of IL-15 into the culture medium. The IL-15 immunohistochemistry when reviewed by histopathologist, Prof Novelli, was removed from this study.

Figure 4.5 Box plots for non-coeliac patients



Enterocyte cell height measurements after overnight incubation of a pair of non-coeliac duodenal biopsies in organ culture system with medium-only (med), ovalbumin (oval), a peptic-tryptic digest of gluten (PT gluten) and the candidate glutenin epitopes HMW glut04 (high molecular weight glutenin$_{04}$ 721-735) and LMW glt156 (low molecular weight glutenin glt$_{156}$ 44-59). The biopsy morphology was preserved in formal saline for 48 hours and embedded in paraffin wax, after which they were cut 4µm thick, dewaxed and stained with haematoxylin and eosin. At x400 magnification the enterocyte cell height from mid-villus was counted for 30 continuous enterocytes in three different villi. The median, interquartile range and maximum and minimum values are represented in the graphs above. Graphs A and B represent different patients with graph C combining the results of both patients. Median values are compared to medium-only to see if there is any difference. ns=not significant, * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

Table 4.8 Interleukin-15: ELISA quantification of secretory interleukin-15 results in non-coeliac patients

Patient (Marsh)	Medium ELISA (pg/mg)	Ovalbumin ELISA (pg/mg)	PT gluten ELISA (pg/mg)	HMW $glut_{04}$ ELISA (pg/mg)	LMW glt_{156} ELISA (pg/mg)
SM11 (0)	0.04	0.06	0.04	0.02	0.03
SM14 (0)	0.01	0.01	0.02	0.03	0.02

Paired biopsies were left overnight in an organ culture system under five different conditions: medium-only, ovalbumin only as a negative peptide, peptic-tryptic digest of gluten (PT gluten) as a positive control, high molecular weight $glutenin_{04}$ 721-735 (HMW glut04) and low molecular weight glutenin glt_{156} 44-59 (LMW glt156). Secretory IL-15 was measured with a commercial high sensitivity ELISA kit from the culture supernatants.

4.6 Discussion

As a method to assess the immunostimulatory potential of various proteins or peptides, the organ culture system benefits from having many more cells present than in a pure T-cell assay, it is closer to *in vivo* studies, and is less invasive than the latter. This system has fallen out of favour in recent times as it is time-consuming and requires rapid setting into the organ culture chamber to prevent damage to the biopsies. However, it is a good method to examine the innate immune response in coeliac disease. Maiuri *et al* (2003) demonstrated the toxicity of A-gliadin peptide 31-43 in coeliac disease patients using this method after T-cell proliferation assays failed to demonstrate immunostimulatory potential of this peptide.

There is no published data on the effects of glutenin peptides on the innate immune response as measured by the organ culture system. Previous studies have suggested that glutenins are not major T-cell epitopes (Tye-Din 2010, Molberg 2003, Vader 2002a) as well as this thesis in Chapter 3. However, *in vivo* studies point to a huge response by HLA-DQ2 positive coeliac individuals' small intestines when instilled with either a purified mixture of high molecular weight glutenin peptide or recombinant Dy10 and Dx5 fractions (Dewar 2006, Ellis 2006). The HMW glut04 peptide is contained within the Dy10 HMW glutenin subunit.

In this study non-coeliac patients were included in order to demonstrate that these glutenin peptides were not toxic to all patient groups. They demonstrate that they do not have a significant reduction in enterocyte cell height after overnight incubation with both the HMW glut04 and LMW glt156 peptides. Unfortunately there were only a few patients in this group due to ethical constraints.

What this study demonstrates is that there is a significant reduction in enterocyte cell height compared to medium alone or ovalbumin in coeliac as well as refractory coeliac individuals when exposed to the HMW and LMW glutenin peptides tested. Reduction in enterocyte cell height is the earliest damage that can be seen in the coeliac small intestine and from Howdle's work (1981b) has been shown to predict coeliac toxicity. Of interest, three patients, SM4, SM10 and SM15, who had no T-cell response to these glutenin peptides, have a marked response in reduction of enterocyte cell height. This suggests that these glutenin candidate peptides exert their effect on coeliac mucosa independent of T-cells. It is not surprising therefore, that there was no difference seen in the two HLA DQ8 positive

patients, SM4 and SM 8, as the HLA molecule is key to the presentation of antigen to T cells. The changes seen in these studies are not necessarily as a result of damage from T-cell activation.

It is not surprising that the level of secretory interleukin-15 measured by ELISA was low as the majority of Il-15 is membrane bound. It is also a tightly regulated hormone as very low concentrations can have significant effects *in vivo*. There was also tissue present containing target cells for the IL-15 which may well have reacted to the IL-15 secreted rather than letting it spill into the supernantant. Perhaps the measurement of IL-15 expression in these biopsies should have included mRNA expression as it may have been a more accurate way of detecting any subtle changes in very low concentrations.

St Thomas Hospital is privileged to have a number of refractory coeliac patients which had not previously been used in this type of study. Little is known about the pathophysiology of this rare complication of coeliac disease however, IL-15 is known to play an important role. Interestingly, the type 2 refractory coeliac group appear to be gluten-sensitive in the organ culture system. This is something that is not thought to be important in clinical practice. In the type 2 refractory group, the major complication is an enteropathy associated T-cell lymphoma derived from the intra-epithelial compartment (Spencer 1989, Cellier 2000). It is possible that the intraepithelial response to peptides triggers the initial response in coeliac patients rather than the gluten peptides in the lamina propria being deamidated and presented to the gluten-sensitive T-cells.

This study suggests that these glutenin proteins, very different structures to gliadin proteins, may be the first trigger in small intestinal response seen in coeliac disease. The next step in the immune response towards gluten peptides is likely to be the T-cells' response to the deamidated lamina propria gluten peptides. More work is needed to confirm these findings.

Chapter 5: The production of monoclonal antibodies against glutenin epitopes

5.1 Introduction and aims

Gluten-free foods are permitted by law to contain gluten in small amounts. This level in Europe is regulated by Codex Standard. The latest guideline (Codex Stan 118-1979) was amended in 2008 to reduce the permissible gluten content in these foods. The change was ratified in January 2009 and manufacturers had until January 2012 to comply. Any food labelled as "gluten-free" must contain less than 20mg/kg of gluten and any food labelled as "very low gluten" may contain between 21-100mg/kg.

This latest amendment also recommends the use of the "R5 Mendez" enzyme-linked immunosorbent assay (ELISA) method to measure gluten content in foods (Valdés 2003). There are inherent problems with this method of gluten quantification. The antibody used detects a pentapeptide, QQPFP, found in rye secalins but also commonly found in wheat gliadins and barley hordeins. However, it fails to measure the glutenin fraction found in wheat, and similar proteins in rye and barley. It also does not measure oat avenins which are immunostimulatory in a minority of coeliac individuals. In order to compensate for the lack of detection of glutenins, the quantity of gliadin measured is multiplied by a factor of 2: gluten=2(gliadin). This can inaccurately estimate total gluten content. Between wheat varieties there is a difference in the ratio of gliadins to glutenins present. High pressure liquid chromatography has demonstrated that the ratio of glutenin to gliadin varies between 0.2 to 5.8 times in wheat starch samples (Wieser 2003). If measured using individual antibodies to a subunit of HMW glutenin, Dy10, as well as antibodies to the immunodominant HLQ-DQ2 α-gliadin epitope, the values obtained for total gluten content were significantly different to the value that would be obtained by multiplying the gliadin value by 2, in a number of gluten-free foods (Ellis 2008b). Therefore, there is a need for a better test to measure gluten content in foods and especially processed foods. The R5 Mendez method can be even more inacurate when measuring processed foods.

Tests for the measurement of gluten in foods must be sensitive enough to detect the lower limit of gluten (20mg/kg) as well as be able to detect hydrolysates present after food processing. The findings of Ellis *et al* (2008b) suggest that both gliadins and glutenins

should be involved in the quantification of gluten. A potential assay which would meet these needs is a “cocktail ELISA”, although this has not yet been designed, due to lack of suitable glutenin monoclonal antibodies. The principle of a “cocktail ELISA” is to use a mix of several antibodies as the primary detection antibodies, such as antibodies to a gliadin peptide, HMWG and LMWG peptides. The resultant value would be closer to the true value of the gluten content in the food. This assay could be further developed into a competition ELISA required to measure hydrolysates in foods.

However, monoclonal antibodies are difficult to produce. The process involves immunising animals with the peptide of interest and assessing the serological response. The animals that display a serological response are culled and the spleens removed. Spleens contain a mixture of T-cells, B-cells, dendritic cells and macrophages. The splenocytes that are B-cells are capable of producing antibodies. However, they are difficult to grow alone. Fused with an immortal cell line, such as mouse myeloma cells, they can produce antibody-secreting cell lines, known as hybridomas. Mice are the ideal vector to use as their small size means that less antigen is required in order to generate an immune response. In this study, mice were on a gluten-free diet to avoid the previously observed oral tolerance of gluten when inoculated with glutenin peptides (Ellis 1989).

One of the main problems encountered in monoclonal antibody work is generating a sufficient immune response to stimulate antibody production in B-lymphocytes to obtain viable hydridoma cell cultures. Injected peptides have to be sufficiently immunogenic for this purpose. Peptides are generally by themselves too small to elicit an immune response. A number of methods have been used to increase the size of the immunogen either by conjugation to a carrier molecule or by the use of multiple antigen peptides (MAP).

Conjugation to a larger carrier molecule can enhance immunogenicity, although this can lead to cross-reactivity and a diversion of antibody production which may need to be screened out later. The choice of carrier protein for conjugation is therefore important. Examples of candidate carrier proteins include purified tuberculin protein derivative (PPD), ovalbumin, bovine serum albumin, keyhole limpet haemocyanine and tetanus toxiod. Peptides in this study were conjugated to purified tuberculin protein derivative (PPD), using methodology that has been used to raise monoclonal antibodies to a toxic gliadin peptide (Ellis 1998). Another group has also managed to raise monoclonal antibodies to a number of gluten epitopes using tetanus toxoid conjugation (Mitea 2008).

Multiple antigenic peptides have a central core of branched poly-lysine residues with peptides of interest attached (Posnet 1989). There are several different types of MAPs based on the size of peptide used. Peptides shorter than 16 amino acids have eight branches whereas longer peptides can elicit a response with four branches. This format ensures the antigen is nearly pure due to the majority of the molecule consisting of the peptide (Tam 1996). Serological responses using MAPs are enhanced compared to immunisation with conjugated peptides (McLean 1991). Several groups managed to raise monoclonal antibodies using the MAP format for the immunogen; however, the numbers of hybridomas seen was small and an even smaller percentage of these were actively secreting antibody (Kamo 1992, Brown 2000).

To increase the immunological response, an adjuvant was used which also controlled a slower release of antigen into the mouse. Freund's adjuvant is the most common adjuvant used for research. It is a water-in-oil emulsion prepared with non-metabolisable oils; complete Freund's adjuvant (CFA) contains killed *Mycobacterium Tuberculosis* whereas incomplete Freund's adjuvant (IFA) does not. Freund's is one of the best adjuvants for stimulating strong and prolonged immune responses. However, the principle disadvantage of Freund's adjuvant is that it can evoke very aggressive and persistent granulomas in the mouse. To minimise these side effects the primary injection is done in CFA with all subsequent injections in IFA.

Chapter 4 demonstrates that both HMW glut04 and LMW glt156 peptides stimulated the innate immune system which then caused damage to coeliac disease small intestinal biopsies *in vitro*. At present there is no direct measurement of glutenin in gluten estimation using the "R5 Mendez" ELISA method. The aim of this particular study was to raise monoclonal antibodies towards HMW $glut_{04}$ and LMW glt_{156} peptides using Balb-C mice in order to facilitate the measurement of glutenin component of foods by ELISA. This would allow a more accurate measurement of total gluten in foods, with the quantification of glutenins and gliadins.

5.2 Methods

Both HMW glut04 and LMW glt156 immunisation schedules ran in parallel to each other. Mice were immunised in groups of three using the protocol as described in section 2.7. These three mice were used to allow for variation in the immune response and for any adverse reactions to the Freund's adjuvant potentially requiring culling. If the peptide used was conjugated to PPD, a BCG immunisation was carried out 2 weeks prior to immunisation with peptide in order to sensitise the mouse to PPD. The first injection was in complete Freund's adjuvant (CFA) and a month later the second one was in incomplete Freund's adjuvant (IFA). A tail bleed was performed 10-14 days after the IFA injection to assess the serological response (see section 2.7.4).

The ELISA methodology used in this study was an established method already successfully running in the department. Initially the serum responses were screened on ELISA plates coated with Frazer's Fraction III, a soluble peptic-tryptic digest fraction of gluten (see sections 1.9.1 and 2.7.4). However, due to unexpected reduced responses they were subsequently tested on peptic-tryptic digest of industrial gluten and recombinant peptic-tryptic digested Dy10, a HMW glutenin subunit containing HMW $glut_{04}$, or LMW glutenin MAP. Negative control serum was obtained from Balb-C mice at the time of cull, having previously been immunised with HLA DQ8 immunodominant peptide and found not to have mounted a serological response. The resultant optical density from the serum was multiplied by a factor of 2 in order to have a cut-off value for negative serum response. Positive control for HMW glut04 was serum of mice previously successfully immunised with recombinant HMW Dy10 by Dewar (2009) as part of his MD thesis. The HMW Dy10 contains the HMWglut04 peptide being tested here. There was no positive control serum for the LMW glutenin peptide on test as none was available.

Mice that demonstrated a good serological response, at least 1:6400 titre on ELISA (Ellis 1989), were given an intravenous injection of peptide (50µg dissolved in 100µl 0.9% saline), between 2 and 10 weeks after the tail bleed as per home office regulations. They were culled using cervical dislocation three to five days later where the spleen was dissected and blood was collected (see section 2.7.6).

A comparison of immunisation schedules for the different methods used in other papers mentioned in this chapter is summarised in table 5.1. Most of the other authors use a more aggressive intraperitoneal injection rather than the subcutaneous route for adjuvant

immunisation. The number of injections used to immunise mice using adjuvant also ranges from 4 (Spaenij-Dekking 2004) to 9 (Kamo 1992), as opposed to the 2 used in this schedule. Brown (2002) also uses 4 injections as an intravenous "booster" rather than the one used in this study.

Table 5.1 Comparison of immunisation schedule in the production of monoclonal antibody

Method	**Format of antigen**	**Dose/ mouse**	**Method of injection**	**1st Injection**	**Subsequent injection(s)**	**Tail bleed**	**Booster-no adjuvant**	**Hybridomas cultured**
This study	MAP and PPD conjugate	150 and 300µg in Balb-C mice	sc	CFA BCG primer 2 weeks prior to PPD	IFA a month later	10-14 days after IFA	50µg 3-5 days prior to cull	culture
Spaenij-Dekking 2004 (method for Mitea 2008)	TTd conjugate	150 µg in Balb-C mice	ip	CFA	IFA at 1,2 and 3 months	none	none	culture
Kamo 1992	MAP and KLH conjugate	30 and 50µg Balb-C mice	ip	30µg IFA	30µg IFA 3 weekly for 21 weeks then for 50µg IFA weekly for 3 weeks	none	50µg 4 days prior to cull, 1 week after the last IFA	ascites
Brown 2000	MAP	10 and 50µg Balb-C mice	ic and ip	10µg CFA	10µg ic week 1 and 3, 50µg ic week 7 and 10	After 2nd IFA injection	200µg 4,3,2,1 day prior to cull	ascites

MAP multiple antigen peptide, PPD purified tuberculin protein derivative, TTd tetanus toxoid, KLH keyhole limpet haemocyanin, sc subcutaneous, ip intraperitoneal, im intramuscular, ic intracutaneous, CFA complete Freund's adjuvant, IFA incomplete Freund's adjuvant, BCG Bacille Calmette-Guérin.

5.3 Peptides

The high and low molecular weight glutenin peptides used for this study were chosen as a result of studies published prior to the start of this research, covered in section 1.12. Van de Wal (1999) demonstrated a region within the HMWG protein, HMW $glut_{04}$, with higher activity using T-cell clones from a single patient. The peptide configuration with the second highest activity was used as this was the more stable peptide to make, according to protein chemists from GenScript (New Jersey, USA). The peptide used in MAP had to be changed as the chemists from Cambridge Peptides (Birmingham, UK) considered the original peptide, QGQQGYYPTSPQQSG, would form ring structures in MAP format and would be difficult to make. The peptide QPGQGQQGYYPTSPQ was used as a compromise, having the 5^{th} highest activity in van de Wal's work. The LMW glt_{156} peptide used is known to stimulate 4 out of 16 T-cell clones in children (Vader 2002a).

Cambridge Peptides supplied the multiple antigenic peptides while GenScript provided the peptides which were then conjugated to purified protein derivative from Statens Serum Institut (Copenhagen, Denmark).

The bold amino acids are the minimum B-cell stimulatory epitopes while the underlined amino acids are the minimum T-cell stimulatory epitopes in humans (Mitea 2008).

Multiple antigenic peptide of HMW glut04 (QPG**QGQQGYYP**TSPQ) x8

Multiple antigenic peptide of LMW glt156 (**PPFSQQ**Q**QSPF**SQQQQ) x8

Amino acids 721-735 of HMW glutenin, HMW glutenin 04 (HMW glut04) conjugated to purified tuberculin protein derivative **QGQQGYYP**TSPQQSG

Amino acids 44-59 of LMW glutenin LMW glutenin 156 (LMW glt156) conjugated to purified tuberculin protein derivative **PPFSQQ**Q**QSPF**SQQQQ

5.4 Results

5.4.1 High molecular weight peptide immunisation: screening results

Table 5.2 shows the serological responses as obtained from the tail bleeds (see section 2.7.5) in the first three mice immunised with HMW glut04 as multiple antigen peptide (MAP) at a dose of 300µg per mouse. Initially the serological response was assessed on an ELISA plate coated with Frazer's Fraction III (25µg/ml in carbonate buffer). As the titres were unexpectedly lower than anticipated, the same serum was tested on plates coated with a peptic-tryptic digest (PT) of the recombinant HMW Dy10 subunit which contained the peptide HMW glut04. This demonstrated an augmentation in the titre of antibody detected in all the mice.

As all three mice had a positive result on PT Dy10 coating antigen ELISA. These three mice were immunised for hybridoma formation (see section 5.4.3, 2.7.6, 2.7.7, 2.7.8 and 2.7.9).

Due to poor hybridoma formation (see section 5.4.3), a further two mice were immunised with HMW glut04 MAP rather than three. This was due to a reduced litter size from the female Balb-C mouse and the need for parallel experiments with the LMW glt156 MAP immunisation. Sera were tested with both PT Dy10 and PT gluten to see if there was any change in the antibody titre measured. The results are shown in table 5.3.

Table 5.2 ELISA screening of serological response to high molecular weight glutenin 04 721-735 (HMW glut04) multiple antigenic peptide (MAP) immunisations in Balb-C mice numbers 1-3

Mouse	**Format of immunogen**	**Weight of immunogen/mouse**	**ELISA coating antigen**	**Titre**
1	HMW $glut_{04}$ MAP	300µg	FFIII 25µg/ml	1:3200
			PT Dy10 3.8µg/ml	1:25600
2	HMW $glut_{04}$ MAP	300µg	FFIII 25µg/ml	1:3200
			PT Dy10 3.8µg/ml	1:12800
3	HMW $glut_{04}$ MAP	300µg	FFIII 25µg/ml	1:3200
			PT Dy10 3.8µg/ml	1:12800
Negative control	HLA DQ8 MAP	300µg	FFIII 25µg/ml	1:400
			PT Dy10 3.8µg/ml	1:400
Positive control	HMW Dy10 PPD conjugate	100µg	FFIII 25µg/ml	1:12800
			PT Dy10 3.8µg/ml	1:25600

FFIII Frazer's fraction III, PPD purified proten derivative, (PT) Dy10 (peptic-tryptic digest of) recombinant Dy10 HMW glutenin subunit containing HMW glut04. Adult Balb-C mice, on a gluten-free diet, were inoculated with MAP format immunogen suspended in complete Freund's adjuvant and a month later inoculated with MAP format immunogen suspended in incomplete Freund's adjuvant. Tail bleed taken 14 days after incomplete Freund's adjuvant subcutaneous injection with immunogen in MAP format and tested on both FFIII and PT Dy10 HMW subunit as coating antigen for ELISA. A positive result was considered a titre greater than 1:6400 and highlighted in grey.

Table 5.3 ELISA screening of serological response to high molecular weight glutenin 04 721-735 (HMW glut04) multiple antigenic peptide immunisations in Balb-C mice numbers 10 and 11

Mouse	Format of immunogen	Weight of immunogen/mouse	ELISA coating antigen	Titre
10	HMW $glut_{04}$ MAP	300µg	PT Dy10 3.8µg/ml	1:400
			PT gluten 25µg/ml	1:400
11	HMW $glut_{04}$ MAP	300µg	PT Dy10 3.8µg/ml	1:400
			PT gluten 25µg/ml	1:400
Negative control	HLA DQ8 MAP	300µg	PT Dy10 3.8µg/ml	1:400
			PT gluten 25µg/ml	1:400
Positive control	HMW Dy10 PPD conjugate	100µg	PT Dy10 3.8µg/ml	1:25600
			PT gluten 25µg/ml	1:12800

MAP multiple antigen peptide, PPD purified protein derivative, (PT) Dy10 (peptic-tryptic digest of) recombinant Dy10 HMW glutenin subunit containing HMW glut04, PT gluten peptic-tryptic digest of industrial gluten. Adult Balb-C mice, on a gluten-free diet, were inoculated with MAP format immunogen suspended in complete Freund's adjuvant and a month later inoculated with MAP format immunogen suspended in incomplete Freund's adjuvant. Tail bleed taken 14 days after incomplete Freund's adjuvant subcutaneous injection with immunogen in MAP format and tested on both PT gluten and PT Dy10 HMW subunit as coating antigen for ELISA. A positive result was considered a titre greater than 1:6400 therefore none of the results from the inoculated mice were considered to be positive.

As the results with the MAP immunogen immunisations were disappointing, further immunisation was undertaken using HMW glut04 peptide conjugated to purified tuberculin protein derivative (PPD). The method had previously produced successful antibodies in this laboratory (Ellis 1993, 1994b, 1998). This required the immune system to be sensitised to the tuberculin protein with BCG vaccination two weeks prior to the scheduled

immunisation with CFA (see section 2.7.3). The results of these immunisations are shown in table 5.4.

Table 5.4 ELISA screening of serological response to high molecular weight glutenin 04 721-735 (HMW glut04) purified tuberculin protein derivative (PPD) conjugate immunisations in Balb-C mice numbers 15 and 16

Mouse	**Format of immunogen**	**Weight of immunogen/mouse**	**ELISA coating antigen**	**Titre**
15	HMW glut04 PPD conjugate	300µg	PT Dy10 3.8µg.ml	1:100
			PT gluten 25µg/ml	1:100
16	HMW glut04 PPD conjugate	300µg	PT Dy10 3.8µg.ml	1:100
			PT gluten 25µg/ml	1:100
Negative control	HLA DQ8 MAP	300µg	PT Dy10 3.8µg/ml	1:100
			PT gluten 25µg/ml	1:100
Positive control	HMW Dy10 PPD conjugate	100µg	PT Dy10 3.8µg/ml	1:25600
			PT gluten 25µg/ml	1:12800

PPD purified tuberculin protein derivative, (PT) Dy10 (peptic-tryptic digest of) recombinant Dy10 HMW glutenin subunit containing HMW glut04, PT gluten peptic-tryptic digest of industrial gluten. Adult Balb-C mice, on a gluten-free diet, were inoculated with MAP format immunogen suspended in complete Freund's adjuvant and a month later inoculated with MAP format immunogen suspended in incomplete Freund's adjuvant. Tail bleed taken 14 days after incomplete Freund's adjuvant subcutaneous injection with immunogen in PPD-conjugate format and tested on both PT gluten and PT Dy10 HMW subunit as coating antigen for ELISA. A positive result was considered a titre greater than 1:6400 therefore none of the results from the inoculated mice were considered to be positive.

None of these results were considered to be positive therefore, further mice pairs were immunised with the HMWglut04 PPD conjugates with the results shown in table 5.5.

Table 5.5 ELISA screening of serological response to high molecular weight glutenin 04 721-735 (HMW glut04) purified tuberculin protein derivative (PPD) conjugate immunisations in Balb-C mice numbers 22 and 23

Mouse	Format of immunogen	Weight of immunogen/mouse	ELISA coating antigen	Titre
22	HMW glut04 PPD conjugate	300µg	PT Dy10 3.8µg.ml	1:100
			PT gluten 25µg/ml	1:100
23	HMW glut04 PPD conjugate	300µg	PT Dy10 3.8µg.ml	1:100
			PT gluten 25µg/ml	1:100
Negative control	HLA DQ8 MAP	300µg	PT Dy10 3.8µg/ml	1:100
			PT gluten 25µg/ml	1:100
Positive control	HMW Dy10 PPD conjugate	100µg	PT Dy10 3.8µg/ml	1:51200
			PT gluten 25µg/ml	1:25600

PPD purified tuberculin protein derivative, (PT) Dy10 (peptic-tryptic digest of) recombinant Dy10 HMW glutenin subunit containing HMW glut04, PT gluten peptic-tryptic digest of industrial gluten. Adult Balb-C mice, on a gluten-free diet, were inoculated with PPD-conjugated immunogen suspended in complete Freund's adjuvant and a month later inoculated with PPD-conjugated immunogen suspended in incomplete Freund's adjuvant. Tail bleed taken 14 days after incomplete Freund's adjuvant subcutaneous injection with immunogen in PPD-conjugate format and tested on both PT gluten and PT Dy10 HMW subunit as coating antigen for ELISA. A positive result was considered a titre greater than 1:6400 therefore none of the results from the inoculated mice were considered to be positive.

Unfortunately there was a wild mouse infestation in the animal house during this experiment and therefore all subsequent experiments had to be abandoned. In conclusion, none of these results were positive.

5.4.2 Low molecular weight peptide immunisation screening results

In parallel with the HMW immunisation schedule (section 5.4.1), Balb-C mice were also being immunised with LMW glt156 as the immunogen. The first immunogens, as for the HMW glut04 peptides, were in MAP format. Table 5.6 shows the serological response as obtained from the tail bleed (see section 2.7.4) in the first three mice immunised with LMW glt156 as multiple antigen peptide (MAP) at a dose of 300µg per mouse. Initially the serological response was assessed on an ELISA plate coated with Frazer's Fraction III (25µg/ml in carbonate buffer) but as the titres were unexpectedly lower than anticipated the same serum was tested on plates coated with the MAP LMW peptide (50µg/ml in 60% ethanol) as there was no other LMW glutenin protein available.

Table 5.6 ELISA screening of serological response to low molecular weight glutenin 156 44-59 (LMW glt156) multiple antigenic peptide immunisations in Balb-C mice numbers 4-6

Mouse	Format of immunogen	Weight of immunogen/mouse	ELISA coating antigen	Titre
4	LMW glt_{156} MAP	300µg	FFIII 25µg/ml	1:6400
			LMW glt_{156} MAP 50µg/ml	1:102400
5	LMW glt_{156} MAP	300µg	FFIII 25µg/ml	1:6400
			LMW glt_{156} MAP 50µg/ml	1:409600
6	LMW glt_{156} MAP	300µg	FFIII 25µg/ml	Died
Negative control	HLA DQ8 MAP	300µg	FFIII 25µg/ml	1:400
			LMW glt_{156} MAP 50µg/ml	1:400

LMW glt156 MAP low molecular weight glutenin156 multiple antigenic peptide, FFIII Frazer's fraction III. . Adult Balb-C mice, on a gluten-free diet, were inoculated with MAP format immunogen suspended in complete Freund's adjuvant and a month later inoculated with MAP format immunogen suspended in incomplete Freund's adjuvant. Tail bleed taken 14 days after incomplete Freund's adjuvant subcutaneous injection with immunogen in MAP format and tested on both FFIII and LMW glt_{156} MAP as coating antigen for ELISA. A positive result was considered a titre greater than 1:6400 and highlighted in grey.

The initial result on FFIII coating plate suggested there was a good serological response to the LMW glt156 MAP immunogen. As mouse 6 had died prior to the tail bleed it was felt that a dose of 300 μg in a mouse may be too toxic. Both mouse 4 and mouse 5 that survived went on to subsequent immunisation for hybridoma formation (see section 5.4.3). The serological response to subsequent immunisations with LMW glt156 MAP were repeated with half the dose of MAP and the results displayed in table 5.7.

Table 5.7 ELISA screening of serological response to reduced dose low molecular weight glutenin 156 44-59 (LMW glt156) multiple antigenic peptide (MAP) immunisations in Balb-C mice numbers 7-9

Mouse	**Format of immunogen**	**Weight of immunogen/mouse**	**ELISA coating antigen**	**Titre**
7	LMW glt_{156} MAP	150μg	LMW glt_{156} MAP 50μg/ml	1:12800 Died 8 days after tail bleed
8	LMW glt_{156} MAP	150μg	LMW glt_{156} MAP 50μg/ml	1:102400
9	LMW glt_{156} MAP	150μg	LMW glt_{156} MAP 50μg/ml	1:12800
Negative control	HLA DQ8 MAP	300μg	LMW glt_{156} MAP 50μg/ml	1:400

LMW glt156 MAP low molecular weight glutenin156 multiple antigenic peptide. Adult Balb-C mice, on a gluten-free diet, were inoculated with MAP format immunogen suspended in complete Freund's adjuvant and a month later inoculated with MAP format immunogen suspended in incomplete Freund's adjuvant. Tail bleed taken 14 days after incomplete Freund's adjuvant subcutaneous injection with immunogen in MAP format and tested on LMW glt_{156} MAP as coating antigen for ELISA, FFIII was not used as it was felt at the time not to add any further value. A positive result was considered a titre greater than 1:6400 and highlighted in grey.

These results suggested that even at half the dose of immunogen the LMW glt156 was capable of evoking a good serological response with a further mouse dying soon after the tail bleed prior to subsequent immunisation for hybridoma formation (see section 5.4.3).

Three further Balb-C mice were immunised with LMW glt156 MAP immunogen as initial hybridoma formation experiments failed to produce a hybridoma colony that secreted the

desired monoclonal IgG antibodyto LMW glt156 peptide (see section 5.4.3). The serological responses to these immunisations are shown in table 5.8.

Table 5.8 ELISA screening of serological response to reduced dose low molecular weight glutenin 156 44-59 (LMW glt156) multiple antigenic peptide (MAP) immunisations in Balb-C mice numbers 12-14

Mouse	**Format of immunogen**	**Weight of immunogen/mouse**	**ELISA coating antigen**	**Titre**
12	LMW glt_{156} MAP	150µg	LMW glt_{156} MAP 50µg/ml	un-interpretable
			PT gluten 25µg/ml	1:800
13	LMW glt_{156} MAP	150µg	LMW glt_{156} MAP 50µg/ml	un-interpretable
			PT gluten 25µg/ml	1:800
14	LMW glt_{156} MAP	150µg	Culled as skin problem due to CFA	
Negative control	HLA DQ8 MAP	300µg	LMW glt_{156} MAP 50µg/ml	un-interpretable
			PT gluten 25µg/ml	1:100

LMW glt156 MAP low molecular weight glutenin156 multiple antigenic peptide, PT gluten peptic-tryptic digest of whole gluten; un-interpretable means that there is no decrease in optical density with the doubling dilutions. Adult Balb-C mice, on a gluten-free diet, were inoculated with MAP format immunogen suspended in complete Freund's adjuvant and a month later inoculated with MAP format immunogen suspended in incomplete Freund's adjuvant. Tail bleed taken 14 days after incomplete Freund's adjuvant subcutaneous injection with immunogen in PPD-conjugate format and tested on both PT gluten and LMW glt156 MAP as coating antigen for ELISA. A positive result was considered a titre greater than 1:6400 therefore none of the results from the inoculated mice were considered to be positive.

Unfortunately mouse 14 had to be culled after the first injection with CFA as it developed granulomas. The serologic repsonse of mice 12 and 13 were difficult to interpret since there was no decrease in optical denisty on ELISA plates. A possible explanation for this could be that the branches of the LMW glt156 peptide in the MAP format trapped the antibodies thereby not allowing successful washing of unbound antibody off the plate. It should be

noted that the MAP was two years old at the time of testing the mouse sera for serological response. Hence it is possible that the structure of the MAP was compromised, or lost, causing problems in interpretation of ELISA results.

As the results with the MAP immunogen form were disappointing, further immunisation was undertaken using LMW glt156 peptide conjugated to PPD. This required the immune system to be sensitised to the tuberculin protein with BCG vaccination 2 weeks prior to the scheduled immunisation with CFA (see section 2.7.3). The results of these immunisations are shown in table 5.9

Table 5.9 ELISA screening of serological response to low molecular weight glutenin glt156 44-59 (LMW glt156) tuberculin-purified protein derivative (PPD) conjugate immunisations in Balb-C mice numbers 17-19.

Mouse	**Format of immunogen**	**Weight of immunogen/mouse**	**ELISA coating antigen**	**Titre**
17	LMW glt_{156} PPD conjugate	150µg	PT gluten 25µg/ml	1:100
			LMW glt_{156} MAP 50µg/ml	un-interpretable
18	LMW glt_{156} PPD conjugate	150µg	PT gluten 25µg/ml	1:100
			LMW glt_{156} MAP 50µg/ml	un-interpretable
19	LMW glt_{156} PPD conjugate	150µg	PT gluten 25µg/ml	1:100
			LMW glt_{156} MAP 50µg/ml	un-interpretable
Negative control	HLA DQ8 MAP	300µg	PT gluten 25µg/ml	1:100
			LMW glt_{156} MAP 50µg/ml	un-interpretable

LMW glt156 PPD conjugate low molecular weight glutenin 156 purified tuberculin protein conjugate, PT gluten peptic-tryptic digest of whole gluten; un-interpretable means that there is no decrease in optical density with the doubling dilutions.Adult Balb-C mice, on a gluten-free diet, were inoculated with PPD-conjugated immunogen suspended in complete Freund's adjuvant and a month later inoculated with PPD-conjugated immunogen suspended in incomplete Freund's adjuvant. Tail bleed taken 14 days after incomplete Freund's adjuvant subcutaneous injection with immunogen in PPD-conjugate format and tested on both PT gluten and LMW glt156 MAP as coating antigen for ELISA. A positive

result was considered a titre greater than 1:6400 therefore none of the results from the inoculated mice were considered to be positive.

There were similar issues with the optical density measured which failed to decrease despite doubling dilution of the sample. For these subsequent experiments washing was done by hand with 4 washes to try to overcome this problem in case it was due to poor washing technique with a multi-channel pipette. This suggested this might indeed be a systemic problem in the ELISA experiment. However, it was also encountered in subsequent experiments shown in table 5.10.

Table 5.10 ELISA screening of serological response to low molecular weight glutenin glt156 44-59 (LMW glt156) tuberculin-purified protein derivative (PPD) conjugate immunisations in Balb-C mice numbers 20 and 21

Mouse	**Format of immunogen**	**Weight of immunogen/mouse**	**ELISA coating antigen**	**Titre**
20	LMW glt_{156} PPD conjugate	150µg	PT gluten 25µg/ml	1:200
			LMW glt_{156} MAP 50µg/ml	un-interpretable
21	LMW glt_{156} PPD conjugate	150µg	PT gluten 25µg/ml	1:100
			LMW glt_{156} MAP 50µg/ml	un-interpretable
Negative control	HLA DQ8 MAP	300µg	PT gluten 25µg/ml	1:100
			LMW glt_{156} MAP 50µg/ml	un-interpretable

LMW glt156 PPD conjugate low molecular weight glutenin 156 purified tuberculin protein conjugate, PT gluten peptic-tryptic digest of whole gluten; un-interpretable means that there is no decrease in optical density with the doubling dilutions. Mice, on a gluten-free diet, were inoculated with PPD-conjugated immunogen suspended in complete Freund's adjuvant and a month later inoculated with PPD-conjugated immunogen suspended in incomplete Freund's adjuvant. Tail bleed taken 14 days after incomplete Freund's adjuvant subcutaneous injection with immunogen in PPD-conjugate format and tested on both PT gluten and LMW glt156 MAP as coating antigen for ELISA. A result of 1:6400 was positive therefore none of these results were positive.

Unfortunately there was a wild mouse infestation in the animal house and therefore all subsequent experiments had to be abandoned. The results from mouse 20 and mouse 21 were uninterpretable.

5.4.3 Result of hybridoma formation

Fusion experiments were undertaken between 2 and 10 weeks after the tail bleeds (see sections 2.7.6, 2.7.7 and 2.7.8) for mice numbers 1-4, 8 and 9 (see sections 5.4.1 and 5.4.2). All of these mice were immunised with MAP immunogen formats. The resultant hybridomas were maintained in culture (see section 2.7.8) and screened for antibody production by ELISA (see section 2.7.9). Unfortunately, mouse number 5 developed a lymphoma, a recognised complication of inbreeding to produce the strain of Balb-C, and had to be culled before any hybridoma fusions could be attempted. The results of the hybridoma fusion experiments are shown in table 5.11 with photographs depicting a hybridoma colony in HT medium in figure 5.1.

Table 5.11 Screening of supernatants from hybridoma fusion experiments from mice 1-4,8 and 9

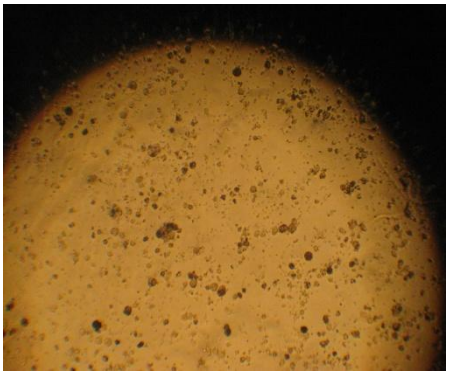
Mouse	Immunogen used	Number of wells containing hybridoma colonies	IgG response	IgM response
1	300µg HMW $glut_{04}$ MAP	0/96	not tested	not tested
2	300µg HMW $glut_{04}$ MAP	0/96	not tested	not tested
3	300µg HMW $glut_{04}$ MAP	2/96	negative	negative
4	300µg HMW $glut_{04}$ MAP	15/96	negative	negative
8	150µg LMW glt_{156} MAP	3/96	negative	1/3 weak +
9	150µg LMW glt_{156} MAP	0/96	negative	negative

The intravenous injection for this experiment used high molecular weight glutenin 04 721-735 (HMW glut04) peptide and low molecular weight glutenin glt156 44-59 (LMW glt156) peptide (50 µg in 100µl 0.9% saline). After fusion, the wells were inspected weekly to screen for any hybridoma formation. Once the hybridomas were identified, 100µl of the medium was removed, added to 20µl HEPES and screened for antibody secretion with PT Dy10 3.8µg/ml as the coating antigen for HMW glutenin antibodies and PT gluten 25µg/ml as the coating antigen for LMW glutenin antibodies. A result was considered positive if the optical density of the sample was at least twice the optical density of HT medium alone. A

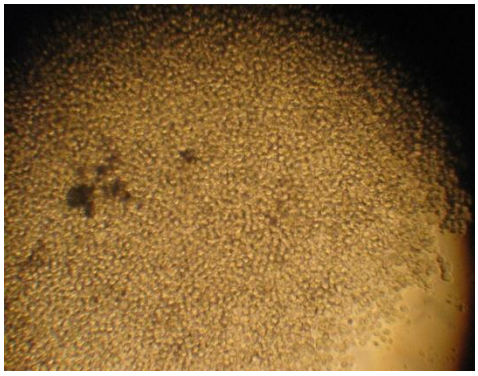
sample was weakly positive if the optical density was one and a half time the optical density of HT medium.

These results suggest that there is no IgG or IgM antibody formation against either candidate glutenin peptide. Mouse 8 had a very low titre of IgM towards the LMW glt156 peptide however this would not have been enough to precipitate and collect for incorporation into an ELISA for the measurement of gluten.

Figure 5.1 Photographs of hybridoma colony and dead splenocytes and myeloma cells in cell culture



Dead splenocytes and myeloma cells



Large hybridoma colony

5.5 Discussion

This research group has previously successfully made monoclonal antibodies to various gliadin peptides (Ellis 1993, 1994b, 1998). However, they experienced considerable difficulties obtaining a serological response in mice to high molecular weight glutenin peptide by conjugating the peptide to purified tuberculin protein (PPD) (Ellis personal communication). This may have been in part due to the small size of peptide being used (Šuligoj 2011) despite conjugation to PPD. Peptide biochemists from the Cambridge MRC Research Laboratory, who first developed the technique of monoclonal antibody formation, suggested a trial of multiple antigenic peptides (MAP) as the immunogen, as at the time the evidence suggested an improved serological response (Brown 2000, Waldron 2002). However, these studies demonstrated a relatively poor hybridoma formation compared to our previous hydridoma production using PPD conjugates (Ellis personal communication). This seems to have been borne out in the present MAP immunisation experiments where initially a good serological response was achieved but very few hybridoma colonies were

seen. Those hybridomas which were produced failed to secrete IgG class antibodies, required for the stability of commercial ELISA tests.

A single researcher performed all the mouse and the ELISA experiments using the same reagents and methodology. The only difference in these MAP format immunisations was the time. Initial mouse experiments with mice 1-9 were done with MAPs that were fresh at the start of the thesis. Subsequent experiments were done towards the end of the thesis with MAPs that were two years old. It was noted that these MAPs formatted peptides were a little more sticky than they had been in initial experiments. Little is known about the stability of MAP-formatted glutenin peptides. It may be that the stability of MAP peptides were affected and this is why there were good serological responses seen in mice 1-9 which were not see in subsequent mice immunisations with MAP-format peptides.

Having tried different methodology using MAP-formatted peptides which did not raise monoclonal antibodies against both HMW glut04 and LMW glt156, immunisations with PPD-conjugated HMW glut04 and LMW glt156 peptides were attempted. The past attempts in the group had failed to raise monoclonal antibodies using PPD conjugated smaller glutenin peptides. It was felt that some of these difficulties could be overcome by increasing the peptide length to try to improve its antigenicity. This was not the case as bourne out by these experiments.

Unfortunately there was a wild mouse infestation in the mouse facility. In every room there are a number of mice kept as health screening mice. They are culled every few months to screen for infections. While the health screening mice did not reveal any evidence of infection it is possible that the mice used in this study were contaminated. This may have meant that the immune response was directed towards the pathogen rather than the glutenin peptides.

It is clear that the LMW glt_{156} in the MAP format is harmful to mice at high dose, with two out of the three mice immunised dying. Mice 1 to 3 as well as 6 to 9 demonstrated higher serological responses as measured by ELISA compared to mice 1 to 3 which were immunised with HMW glut04 MAP. Perhaps the lower dose used was too low to obtain a serological response. It is not clear why LMW glt156 should be harmful to mice.

Another difficulty encountered in the attempt to raise monoclonal antibodies to LMW glt_{156} was the lack of a good testing antigen. For HMW $glut_{04}$ screening there was Dy10 HMW

subunit available containing HMW glut04 peptide, as well as positive mouse sera against this protein to use as a positive control. For screening LMW glt156 there was no similar coating antigen or mouse serum available. Autran *et al* (1987) demonstrated that the percentage of LMW glutenins in two different stains of durum wheat varied from 18% to 27%. The coating antigen for the ELISA plates was PT gluten from industrial gluten: the percentage content of LMW glutenins is unknown. As this study shows, if the percentage LMW glutenin was low, the resultant signal from the ELISA could also be low.

Antibody production requires T-cells to be primed and interact with B-cells. Vasconcelos *et al* (2004) demonstrated that the immunogenicity of B-cell epitopes in MAP format can be augmented by the combination of a universal T-cell epitope in tandem. The known B and T-cell epitopes in these glutenin peptides are human, not necessarily mouse epitopes, and are unlikely to contain a universal T-cell epitope. However, another group has successfully raised monoclonal antibodies to both these glutenin peptides used in this study (Mitea 2008). Their methodology (Spaenij-Dekking 2004) differs considerably to the one used in this study, with much greater number of immunisations, at different sites. Brown *et al* (2000), who raised IgG class antibodies against JS30 (a vitamin K protein which is highly immunogenic) with MAP format, also differed in the methodology used. They used a total of 5 intracutaneous and intraperitoneal injections and had 4 intravenous "booster" injections to gain a small number of IgG secreting hybridomas. However, both methodologies were discarded as they would fail to pass through an animal ethics committee in the UK. Animal experiments in the UK are bound by the 3Rs; Reduction in the number of animals used, Refinement of techniques to minimise discomfort and Replacement of animals with *in vitro* models, where possible. Both studies (Spaenij-Dekking 2004 and Brown 2000) were performed outside the UK, so workers were not bound by the same Home Office rules for use of animals in experiments.

Monoclonal antibodies raised in ascites have been banned and the number of injections has been limited to two by the Home Office. On this basis Kamo's methods (1992, see table 5.1) would not be approved in the UK due to the use of ascites as well as the large number of IFA immunisations.

The use of Freund's adjuvants in the present experiments was strongly discouraged, however available alternatives, such as TiterMax™, are only allowed for a single subcutaneous injection by King's College London present animal ethics committee; this

may not be enough to generate an IgG class of antibody (product literature). As for the purposes of this thesis this class of antibody is required to generate stable ELISA reagents rather than IgM class antibodies the alternatives would not serve.

Future directions could target different approaches. If MAP format was to be used then a universal T-cell epitope, such as tetanus toxoid, could be used in tandem with the peptide. At present this methodology has been used to improve the serological response in the development of vaccines. The use of TiterMax™ instead of Freund's adjuvant, keeping the same protocol, may improve the serologic response seen, but may be difficult to pass the animal ethics committee. These two changes would mean the severity of the animal project would remain as "mild". Another option could include increasing the severity of the project to "moderate" and increasing the number of IFA injections, although this is likely to be met with opposition in the Animal Ethics Committee at King's College London, and the UK Home Office.

Chapter 6: General discussion and future directions

6.1 General discussion

Research into the immunostimulatory potential of glutenin proteins has been hampered in the past by problems obtaining pure glutenin samples. Glutenin reactive T-cell clones were isolated (van de Wal 1999, Vader 2002a). With improved extraction methods (Wieser 2003), Dewar *et al* (2006) were able to demonstrate, with a mixture of chemically purified high molecular weight (HMW) glutenin subunits Dx5, Bx7, By9 and Dy10, T-cell stimulation in 11 out of 17 lines. Recombinant proteins Dy10 and Dx5 were tested by various groups using T-cell lines, Molberg (2003), Ellis (2006), and they found that in a minority of lines these proteins were immunostimulatory. Dewar *et al* (2006) as well as Ellis *et al* (2006) carried out *in vivo* studies instilling their HMW glutenin subunits, chemically purified or recombinant, into the duodena of coeliac patients and found a dramatic flattening of the duodenal mucosa starting an hour after the subunits were infused. These studies suggested that HMW glutenins were immunostimulatory in coeliac patients, although the exact disease-stimulating epitope remains to be characterised.

The evidence for the immunostimulatory potential of low molecular weight (LMW) glutenin proteins was even less well established. Vader *et al* (2002a) tested novel T-cell epitopes in children, using T-cell clones, and found that a small number of clones extracted from a few coeliac individuals responded to two LMW glutenin peptides. These peptides, however, failed to stimulate the T-cell clones isolated from adult coeliac individuals.

In demonstrating that the children do not react to the same epitopes as adults, Vader's work, in 2002(a) started a debate in coeliac research over the possibility that children and adult coeliac individuals respond to different epitopes. A theory has evolved that the range of immunostimulatory epitopes to which coeliac individuals will mount a response is reduced as they age, perhaps due to the focussing of the immune system to these epitopes. In my own thesis, the earlier in culture a sample was tested, the more immunostimulatory it was likely to be. Furthermore, the newly diagnosed coeliacs were more likely to have an

immunostimulatory T-cell response. Both these findings are consistent with the "epitope spreading and focussing" argument.

Tye-Din *et al* (2010) further suggested that both HMW and LMW glutenin peptides were not immunostimulatory to T-cells from the majority of HLA-DQ2 positive coeliac individuals tested. This was a large study measuring peripheral blood lymphocyte interferon-γ secretion from coeliac individuals who had undergone a five-day gluten challenge.

In this thesis, the results presented in Chapter 3 agree with those in Tye-Din's manuscript (2010) suggesting that glutenins are not major T-cell epitopes. T-cell responses are enhanced by deamidation of glutamine to glutamic acid in key QXP motifs (Vader 2002b). In both the HMW and LMW proteins, there are very few of these motifs present and none in the HMW glut04 and LMW glt156 peptides tested. This may explain why T-cells in the majority of coeliac patients tested failed to mount a response to these peptides.

While the evidence was lacking for the HMW glut04 and LMW glt156 glutenin epitopes to be major T-cell epitopes, the results presented in Chapter 4 demonstrated that these peptides are capable of inflicting damage to the mucosa of not only individuals with coeliac disease but also type 2 refractory coeliac disease. This is the first study of its kind using the organ culture system to assess the coeliac toxicity of glutenin peptides in both coeliac disease and type 2 refractory coeliac disease. Exactly what is initiating the mucosal damage is unclear, however, Maiuri's work (2003) highlighted the role of interleukin-15 in potentially triggering the innate immune response. My own work investigates this further.

Chapter 4 demonstrates a process happening in the mucosa leading to a reduction in enterocyte cell height. It is likely to be independant of a T-cell response as three of the coeliac patients in Chapter 4 (SM4, SM10, SM15) failed to mount a T-cell response in Chapter 3 (SD20, SD50, SD63). This must be happening at the mucosal level.

A-gliadin 31-43, the "innate peptide", has an unusual QQQXXXXQQ motif, where XXXX represent any amino acids, not commonly found in the known immunostimulatory gliadin epitopes, but found in the LMW glt156 peptide tested and the HMW glutenin subunits. In HMW glutenins there is a repeating sequence of QQGYY motifs. These amino acid sequences, as well as the lack of QXP motifs to favor deamidation, may well explain why the T-cells do not mount an immune response.

Zimmer *et al* (2010) found that in enterocytes there was delayed endocytic vesicle trafficking and maturation with A-gliadin 31-49 compared to other gliadin pepides. The group found that the "innate peptide" was present in endosomes in the luminal surface of the enterocytes rather than the basolateral surface, required for antigen-presentation to T-cells. They suggest that A-gliadin 31-49 is not presented to T-cells in the lamina propria which explains why there is no T-cell response. However, another possible explanation could be that it is the failure to migrate through the enterocyte that causes up-regulation of IL-15. The peptide structure may well be the cause for the failure of endosomes to migrate through the enterocytes. This in turn may cause stress to the enterocytes which could express MICA on its surface via IL-15 and the enterocyte releases granzyme and perforin from exosomes which ultimately lead to its destruction. This may explain the enterocyte cell height changes seen with the glutenin peptides in Chapter 4.

Chapter 4 further supports the idea that the immune response in the mucosa of coeliac disease is not just an adaptive one but also involves the innate immune system. Previous studies (Sturgess 1994, Fraser 2003) suggest that the activation of an immune response in coeliac disease occurs within the first hour of gluten ingestion. This would favour the innate response being the initial gut mucosal response in coeliac disease with the T-cell response occurring some time later and helping maintain the damage seen on histology. It also demonstrates that when assessing the immune response towards candidate epitopes, a T-cell response alone is insufficient to say that no damage will occur to the mucosa of coeliac individuals.

Following on from this discovery, there was increasing evidence that glutenins should be included when assessing the gluten content in foods for individuals with coeliac disease. This thesis further supports the need to measure both gliadins and glutenins in the measurement of total gluten content in foods for individuals with coeliac disease. At present the way in which gluten is measured does not include a measurement of glutenins, the amounts of which is known to vary among different wheat cultivars and particularly in processed foods. Current assays are using monoclonal antibodies to measure a pentapeptide found in rye. By measuring different gluten components, HMW, LMW glutenins and gliadins, more accurate gluten measurement can be achieved. Unfortunately, due to the stricter experimental animal health regulations in the UK compared to other institutions this has made progress in this area difficult. In particular, the difficulties the regulations cause

have been illustrated in this thesis by the several different attempts to produce monoclonal antibodies.

6.2 Future directions

6.2.1 Monoclonal antibodies production

Because of the stringent regulatory experimental animal health regime alluded to above, monoclonal antibody production in the UK is unlikely to be competitive against its European counterparts. However, a number of alternative possibilities may be found to deal with that situation. Multiple antigenic peptides appear to work better when a universal T-cell epitope, such as tetanus toxoid, is made in tandem with the B-cell epitope peptide. This strategy is currently being evaluated in vaccine design. The use of a different adjuvant such as TiterMax™ to replace Freund's adjuvants might offer some benefit but as the manufacturer cannot guarantee an IgG response after a single injection this would need a change in protocol from "mild" to "moderate" in the animal licence. Incomplete Freund's adjuvant could also be used in this case with an increased number of immunisations.

6.2.2 The role of glutenins in the pathogenesis of coeliac disease

The role of glutenins in the pathogenesis of coeliac disease should be investigated further. A number of interesting points were raised by this thesis and further investigational strategies are suggested below:

1. Improving the organ culture experiments: This thesis has demonstrated the importance of testing candidate epitopes with both a T-cell response as well as an innate immune system response. Messenger RNA (mRNA) expression in the biopsies may be a better way to measure the IL-15 activation. Labelled monoclonal antibodies for HMW glut04 and LMW glt156 could be used with electron microscopy to see if the peptides are found in early endosomes rather than the late endosomes found in the transport of most gliadins. By combining the IL-15 mRNA data with the electron microscopy data, this may help pinpoint the immune pathway involved in the villous atrophy seen on the biopsy specimens. Using Luminex multiplex assays for the analysis of the culture supernatants may increase the understanding of the cytokines secreted. However, because there is tissue present that contain many receptors, it may not be beneficial to clarify the precise pathogenic mechanisms involved.

2. The role of HLA DQ8: Most coeliac epitope studies do not include HLA DQ8 patients as they are less common than HLA DQ2 patients. Mazzarella (2003) demonstrated that these patients have a different immunodominant epitope to HLA DQ2 patients. Vader (2002a)

and van de Wal (1999) both tested HLA DQ8 positive patients with demonstrating T-cell stimulation against HMW glut04 and LMW glt156 peptides. Very little is known on the disease course of these patients. The immunodominant HLA DQ8 epitope is not as immunostimulatory as the HLA DQ2 immunodominant epitope. This could suggest a more "benign" form of coeliac disease. Larger studies with this patient group are required in any epitope immunostimulatory testing. This may require participation between different hospitals and clinicians in the country to obtain sufficient results. It would also necessitate the HLA DQ status to be tested in every coeliac patient which, at present, is not routinely undertaken. This may change if the EPSGHAN diagnostic criteria are changed in the paediatric cohort as currently proposed.

3. Further investigation of epitope focussing: Little is known about the potential change in epitope response in adults at initial diagnosis and then several years later, or those who were diagnosed early in life versus later in their adult life. Another potential study would be to study the epitope response of those patients in their first 6 months of diagnosis in their teenage years or twenties as opposed to those diagnosed in later life. The latter is becoming more common and it is these patients that are more likely to develop refractory coeliac disease. A longitudinal study including both small intestinal T-cell lines and organ culture systems looking at newly-diagnosed coeliac patients, young and older, over many years would be of benefit as little is published on the "epitope focussing" theory. It should include glutenin epitopes as well as the better understood gliadin epitopes to see what happens to the immune response at the beginning of a disease and further into it.

4. The effect of glutenin epitopes on dendritic cells in coeliac disease: Very little is known about the role of dendritic cells in coeliac disease. A recent study suggested that the phenotype of dendritic cells was controlled by the concentration of tissue transglutaminase 2 in the lamina propria (Dalleywater 2012). At low concentrations the dendritic cells acted more like sentinels sampling the antigens available but at higher concentrations these became much better at presenting antigen to the T-cells. We know that these glutenin epitopes are not major T cell epitopes but their effect on tissue transglutaminase concentrations as well as their role in dendritic cells would be interesting to discover.

5. *in vivo* testing in coeliac patients: While *in vitro* testing is helpful to investigate further the immunostimulatory potential of any epitope these are artificially created conditions. In the 1980s Ciclitira *et al* used a Quinton Hydraulic capsule to take biopsies while instilling

into the duodenum of coeliac patients candidate toxic proteins and helped characterise further the coeliac disease epitope toxicity of α, β, γ and ω fractions of wheat gliadin. With the advances in immunology testing this could give real time immunological processes which could be sampled. However, the disadvantages are that it is an invasive technique which requires skilled clinicians and the Quinton capsule is no longer being made and repaired. While there are still a few of these in circulation it will not be long before this technique dies out.

6. The role of glutenins in IBS: Jabri in a recent conference (International Coeliac Disease Symposium, Oslo 2011) suggested that in order to develop coeliac disease both the innate immune response and the adaptive immune response had to be triggered. If it was just the innate response being triggered then IBS-type symptoms on gluten ingestion were experienced. Gluten is a potentially toxic molecule to humans as with its high proline content human proteases find it difficult to cleave these proteins into smaller peptides. The larger the peptide size, the more likely it is to trigger an immune response. Gluten sensitivity is a common complaint amongst irritable bowel syndrome (IBS) sufferers, although they do not develop the villous atrophy seen in coeliac disease. This thesis demonstrates that HMW and LMW glutenin peptides are capable of generating an immune response in not only coeliac disease but also refractory coeliac disease. It would be interesting to put gluten-sensitive IBS patient biopsies in the organ culture system to determine if the triggering of the innate immune reponse by glutenin peptides may be observed.

Some of these potential research projects would be difficult to implement. The Quinton capsule technology and experience is dwindling. It is also more invasive than an upper GI endoscopy and no longer routinely used in the diagnosis of coeliac disease. This makes patient recruitment difficult. Due to the paucity of HLA DQ8 coeliac patients, a multicentre research group would need to be established. This can be costly in time and resources and is unlikely to be easily attainable in a short time period. However, the changes occuring in the paediatric diagnosis may fascilitate this research in the future.

Comparison of the immune repsonse to these glutenin peptides in gluten-senstive IBS against coeliac disease would be interesting. The most useful investigation strategy would be the combination of mRNA IL-15 measurement in organ culture biopsies after overnight incubation with the candidate peptides as well as the monoclonal antibody staining and

electron microscopy of the enterocytes. Commercially-made monoclonal antibodies may fascilitate this potential study. This research could help highlight further areas of interesting research as little is known about the enterocyte interaction with intra-epithelial cells in coeliac disease. This is likely to be the focus of coeliac immunology research groups in the near future and would augment the work already carried out in this thesis.

Appendix I: References

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Appendix II: Materials

T Cell Studies

Autologous Plasma Culture Medium (50ml)

- 5ml autologous, heat inactivated serum
- 0.5ml HEPES (N-2-hydroxyethylpiperazine-N-2ethane sulphonic acid) buffer (H0887, Sigma)
- 0.5ml amphotericin B (0.25mg/ml, P11-001, PAA)
- 50µg Plasmocin (25µg/ml, ant-mpt, Source BioScience) (for prevention of mycoplasma infection)
- 44ml RPMI1640 medium with L-glutamine (E15-840, PAA)

Organ Culture Medium (10ml)

- 8ml RPMI 1640 (E15-840, PAA)
- 0.1ml HEPES (H0887, Sigma)
- 0.1ml Amphotericin B (PAA)
- 10ul Plasmocin (Source BioScience)
- 1.5ml Heat-inactivated foetal calf serum (A15-101, PAA)

Histology

Formal saline (100ml)

- 0.9g sodium chloride (S9888, Sigma)
- 90 ml distilled water
- 10ml formaldehyde (BDH10113, VWR)

1% v/v acid alcohol (100ml)

- 70 ml 74 OP ethanol
- 29ml distilled water
- 1ml concentrated hydrochloric acid (BDH28507, VWR)

Monoclonal work

Carbonate buffer (100ml) pH 9.6

- 159mg sodium carbonate (S2127, Sigma)
- 293mg sodium bicarbonate (S5761, Sigma)
- 100ml distilled water

Double strength antibiotic medium (50ml)

- 46.5ml RPMI 1640 (PAA)
- 2ml Penicillin/Streptomycin (5,000u penicillin, 5,000g streptomycin/ml, 15070063, Invitrogen)
- 0.5ml Gentamicin (10mg/ml, 15710, Invitrogen)
- 1ml Amphotericin (PAA)

Single strength antibiotic medium (50ml)

- 48.25ml RPMI 1640 (PAA)

- 1ml Penicillin/Streptomycin (5000u/5000µl/m, Invitrogen)
- 250µl Gentamicin (10mg/ml, Invitrogen)
- 0.5ml Amphotericin B (PAA)

HAT medium (100ml)

- 20ml Foetal calf serum (A15-101, PAA)
- 2ml HAT (H0262, Sigma)
- 1ml Amphotericin B (PAA)
- 50µl Plasmocin (Source BioScience)
- 77ml RPMI 1640 (PAA)

HT medium (100ml)

- 20ml Foetal cal serum (PAA)
- 2ml HT (H0137, Sigma)
- 1ml amphotericin B (PAA)
- 50µl plasmocin (Source BioScience)
- 77ml RPMI 1640 (PAA)

Saturated ammonium sulfate solution

- 761g ammonium sulfate (A4915, Sigma)
- 1l distilled water

Appendix III: Reagents and Equipment addresses of suppliers

Amersham Biosciences UK Ltd, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, UK

Axis-Shield PoC, Kjelsasveien 161, Oslo, N-0884. Norway

BDH Chemical Ltd, see VWR

Bio-Equip, 304B Sanhang Mansion, 139 Pinjiang Road, Shanghai, 200032, P.R. China

Bio-Optica, via San Faustino 58, I-20134 Milano, Italy

Cambridge Peptides Ltd, 1 Phillip Victor Road, Birmingham, West Midlands, B20 2QB, UK

Clin-Tech Ltd, Unit G, Perrham Works, Merrow Lane, Guilford, Surrey, GU4 7BN, UK

Fisher Scientific UK Ltd, Bishop Meadow Road, Loughborough, Leicestershire LE11 5RG, UK

GenScript USA Inc., 860 Centennial Ave, Piscataway, NJ 08854, USA

Harlan Teklad, Shaws Farm, Blackthorn, Bicester, Oxfordshire, OX25 1TP, UK

Health Protection Agency Culture Collections, Centre for Emergency Preparedness and Response, Porton Down, Salisbury SP4 0JG, UK

Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK

LEO Laboratories Ltd, Longwick Road, Princes Risborough, Buckinhamshire, HP27 9RR, UK

MP Biomedicals, Inc. Rue Geiler de Kayserberg, Illkirch, 67402, France.

Nunc A/S, Kamstrupvej 90, Postbox 280 DK-4000 Roskilde, Denmark

PAA Laboratories, PAA-Strasse 1, 4061 Pashing, Austria

PerkinElmer, Chalfont Road, Seer Green, Buckinghamshire HP9 2FX, UK

R&D Systems Inc, 614 McKinley Place NE, Minneapolis, MN 55413, USA

Receptor Technologies Ltd, 23 Cross Street, Leamington Spa CV32 4PX, UK

Scientific Laboratory Supplies Ltd (SLS), Orchard House, The Square, Hessle, East Riding of Yorkshire, HU13 0AE, UK

Sigma-Aldrich Company Ltd, Fancy Road, Poole, Dorset BH12 4QH, UK

Source BioScience UK Ltd, 1 Orchard Place, Nottingham Business Park, Nottingham, NG8 6PX, UK

Statens Serum Institut (SSI), 5Artillerivej, 2300 Coperhagen S, Denmark

VWR International-Jencons Ltd, Hunter Boulevard, Magna Park, Lutterworth, Leicestershire LE17 4XN, UK

Appendix IV Publications arising from this thesis

Papers

Dewar DH, Donnelly SC, McLaughlin SD, Johnson MW, Ellis HJ, Ciclitira PJ. (2012) Celiac disease: Management of persistent symptoms in patients on a gluten-free diet. World J Gastroenterol 18:1348-56.

Donnelly SC, Ellis HJ, Ciclitira PJ. (2011) Pharmacotherapy and management strategies for coeliac disease. Expert Opin Pharmacother 12:1731-44.

Posters

Donnelly SC, Šuligoj T, Ellis HJ, Ciclitira PJ. (2011) Response of coeliac gluten-sensitive small intestinal T-lymphocytes to glutenin peptides. 14th International Coeliac Disease Symposium Oslo June 2011.

Donnelly SC, Šuligoj T, Ellis HJ, Ciclitira PJ (2011) Immunostimulatory potential of glutenin peptides in celiac disease small intestinal lymphocytes. Gastroenterology 140(5) Supplement 1

Šuligoj T, Donnelly SC, Božič B, Ciclitira PJ, Ellis HJ. (2011) Overcoming poor immunogenicity of gluten peptides using multiple antigenic peptide. Eur. J. Pharm. Sci. 44, Supl 1: 66-67

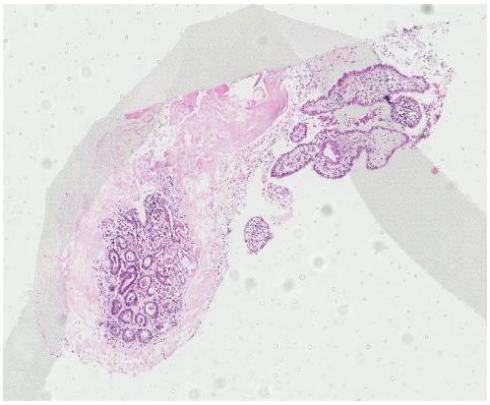
Appendix V: Photographs of some H+E stained slides from Chapter 4

Duodenal biopsies were set in organ culture chambers for overnight incubation with various antigens before fixing in 10% formal saline and embedding in paraffin. They were then satined with H+E.

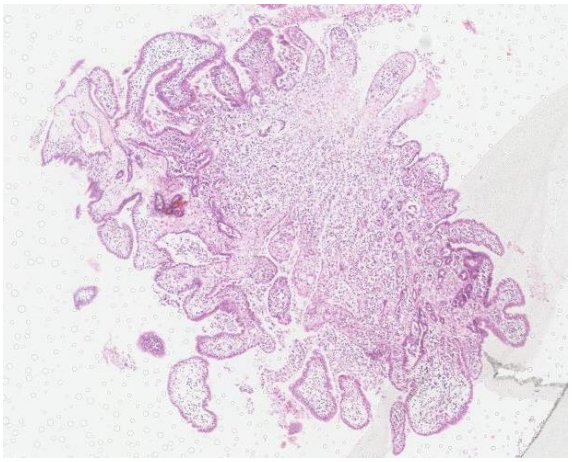
Coeliac patients

SM4: Coeliac patient HLA DQ8 with coeliac disease for 3 years.

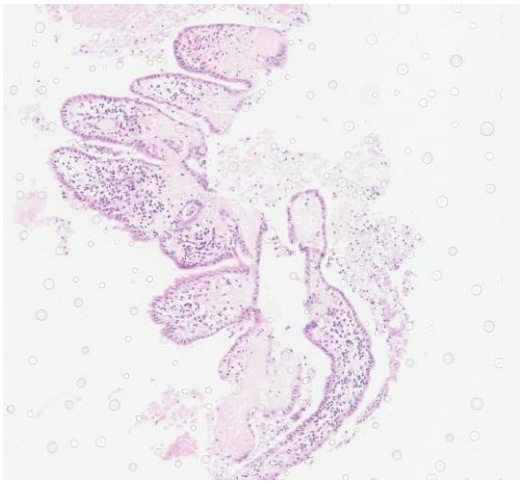
Medium-only magnification x2.5



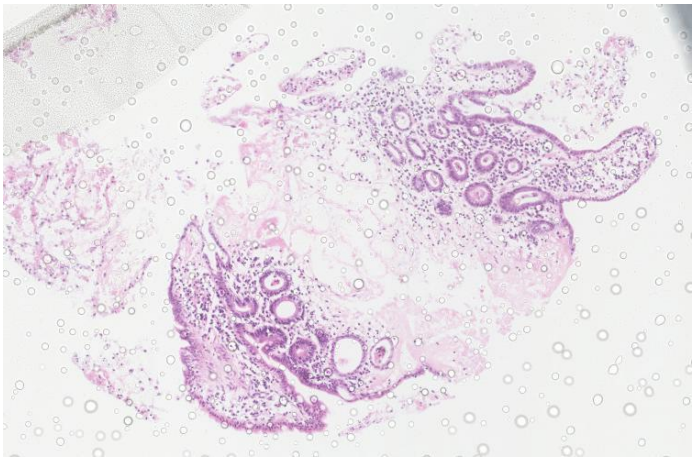
Ovalbumin 1mg/ml magnification x2.5



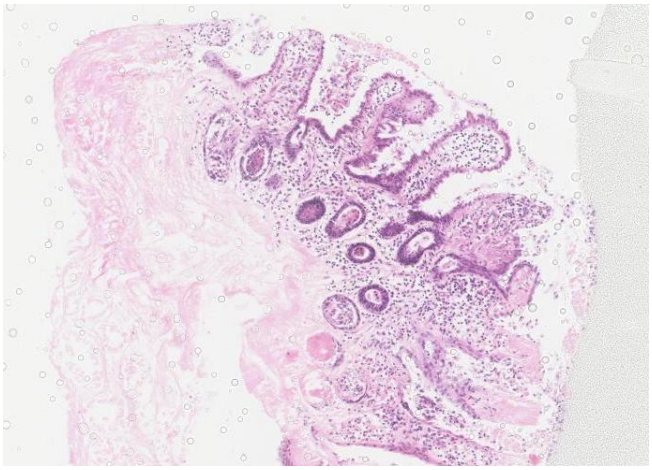
Peptic-tryptic digest of gluten 1mg.ml magnification x5



HMW glut 04 200µg/ml magnification x5

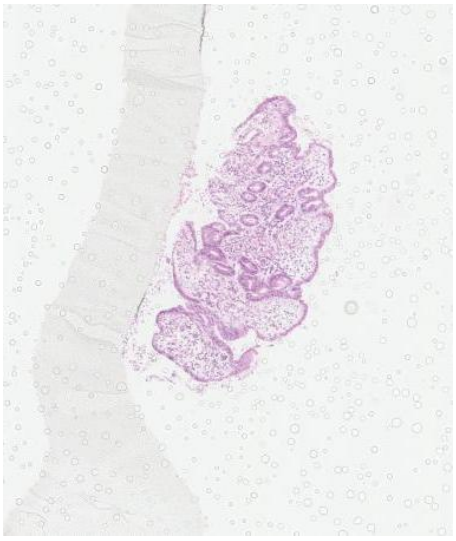


LMW glt156 200µg/ml magnification x5

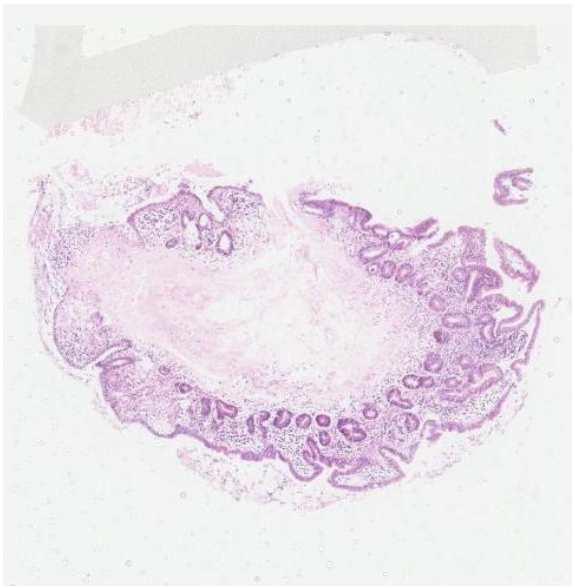


SM5: coeliac patient HLA DQ2 with coeliac disease for 14 years

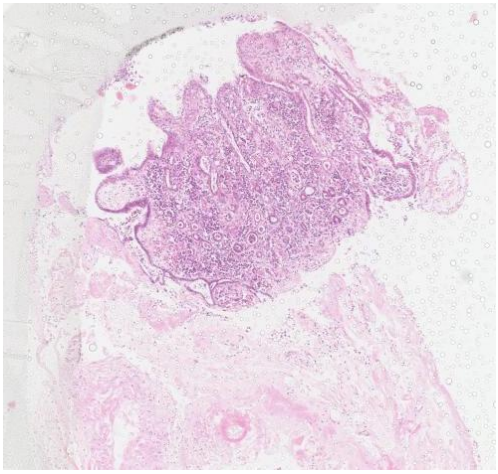
Medium-only magnification x2.5



Ovalbumin 1mg/ml magnification x2.5



PT gluten 1mg/ml magnification x2.5



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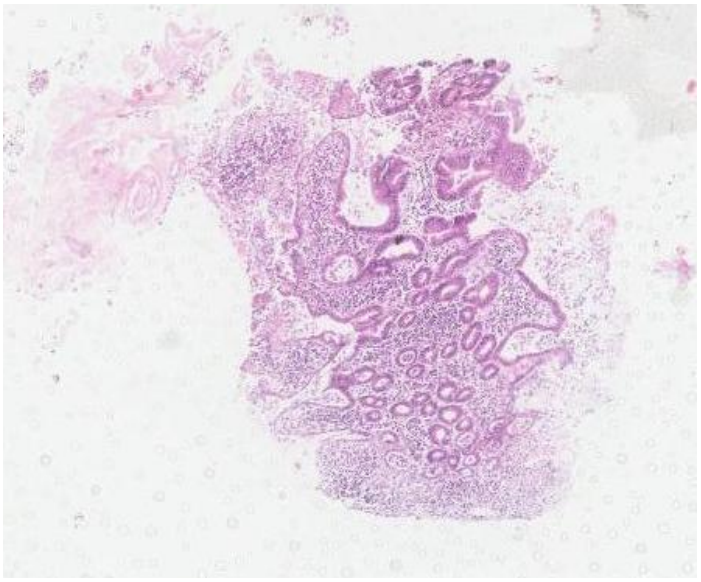
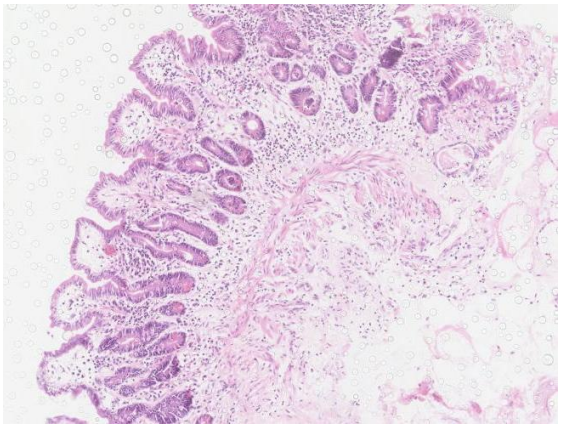
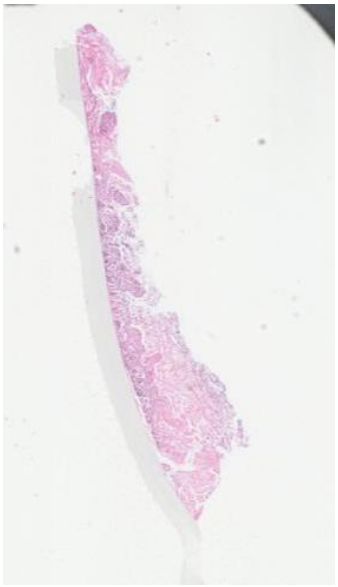


LMW glt156 200µg/ml magnification x5



SM6: Coeliac patient HLA DQ2 with coeliac disease for 4 years

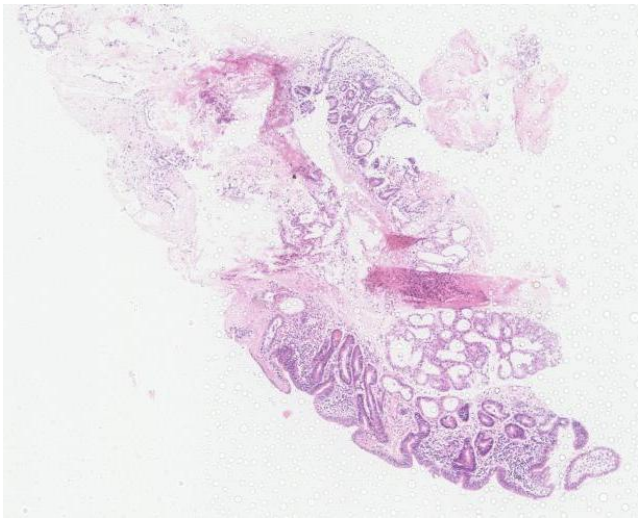
Medium-only magnification x1.07



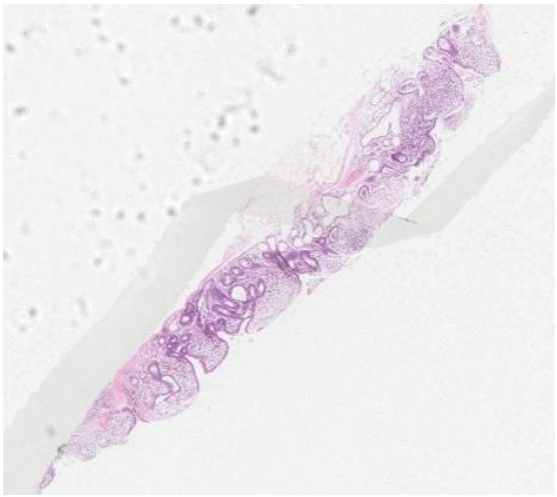
Ovalbumin 1mg/ml magnification x0.88

PT gluten 1mg/ml magnification x2.5

HMW glut04 200µg/ml magnification x2.5

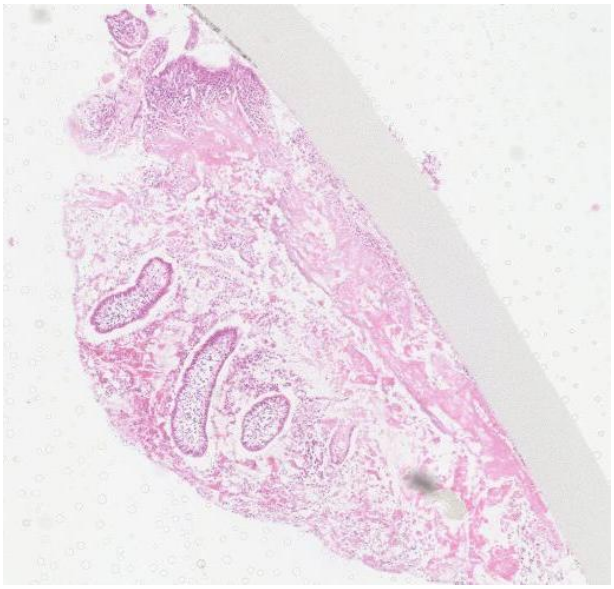


LMW glt156 200µg/ml magnification x 2.5

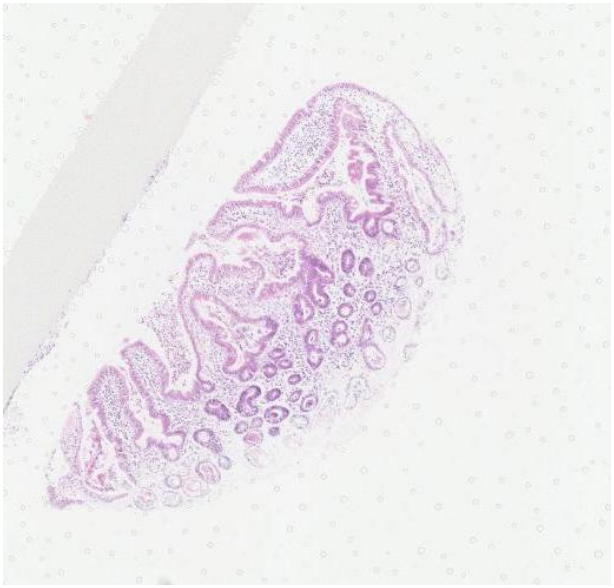


SM9: coeliac patient HLA DQ2 coeliac disease for 11 years

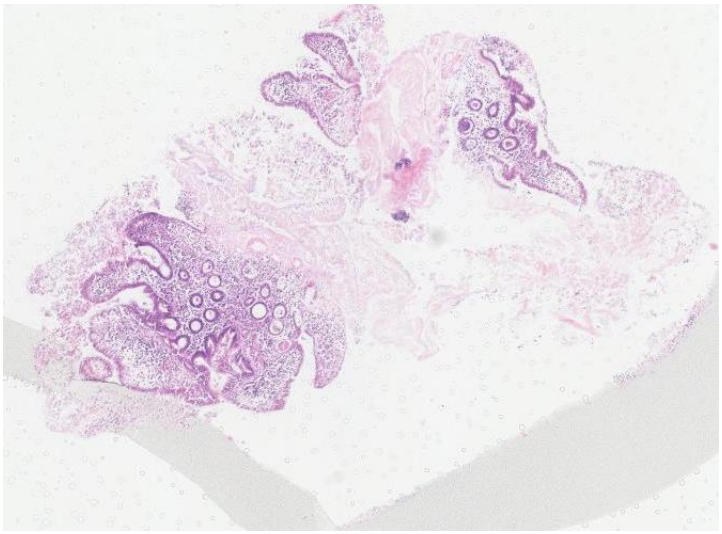
Medium-only magnification x2.5



Ovalbumin 1mg/ml magnification x2.5



PT gluten 1mg/ml magnification x2.5



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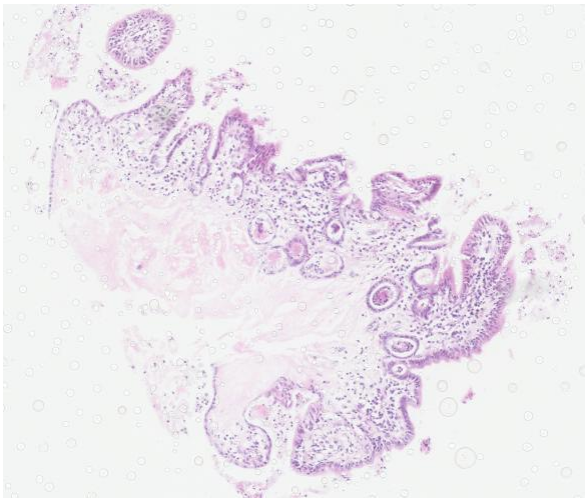

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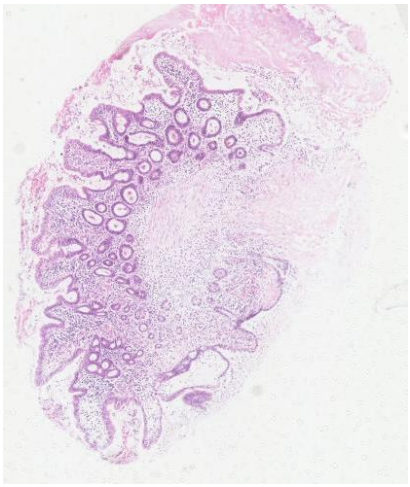
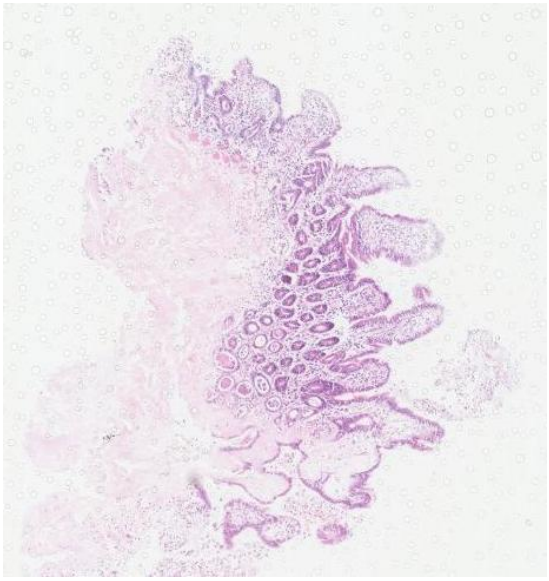
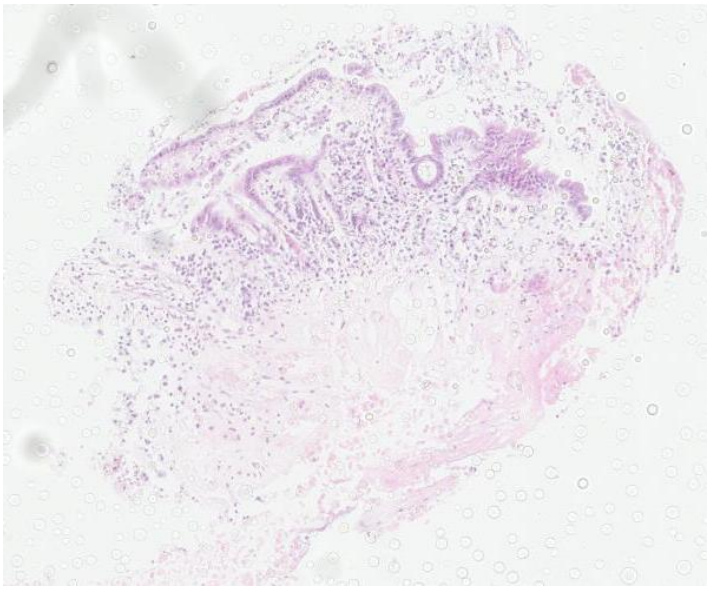


SM10: coeliac patient HLA DQ2 12 year history of coeliac disease

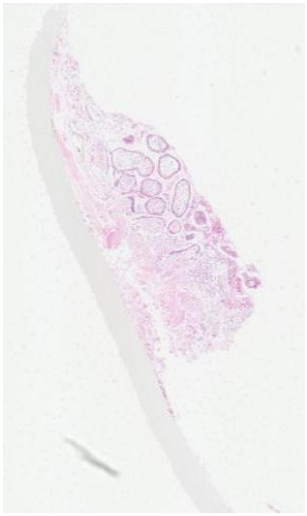
Medium-only magnification x2.5



Ovalbumin 1mg/ml magnification x5



PT gluten 1mg/ml magnification x2.5



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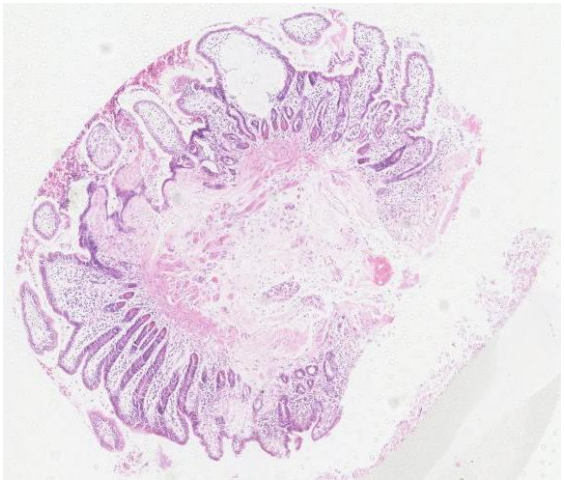
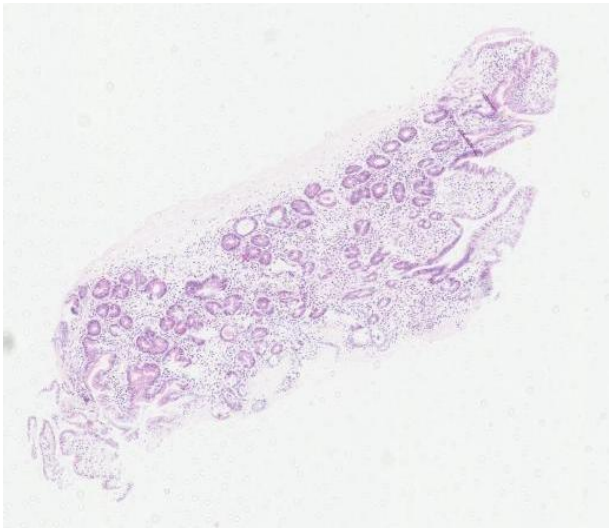
LMW glt156 200µg/ml magnification x2.5



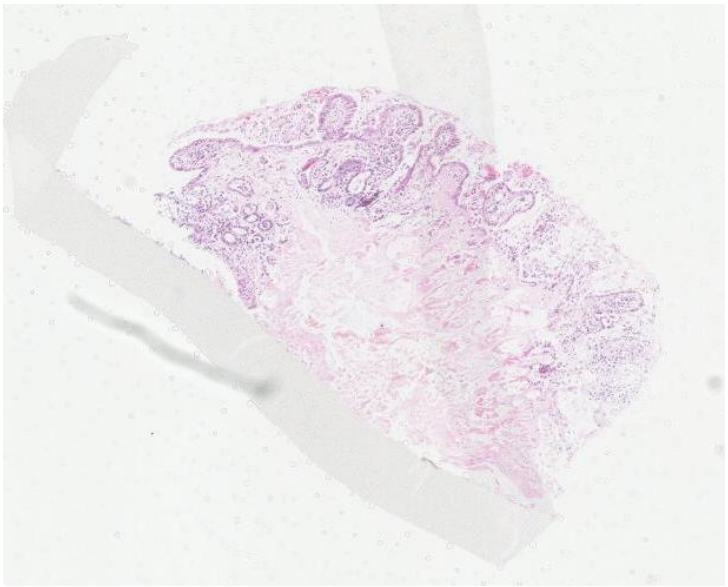
Type 2 refractory coeliac patients

SM7: Type 2 refractory coeliac disease HLA DQ2 coeliac disease for 24 years, RCD for 6 years

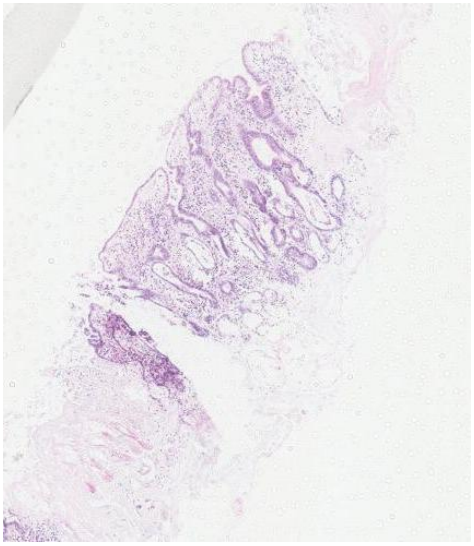
Medium-only magnification x2.5



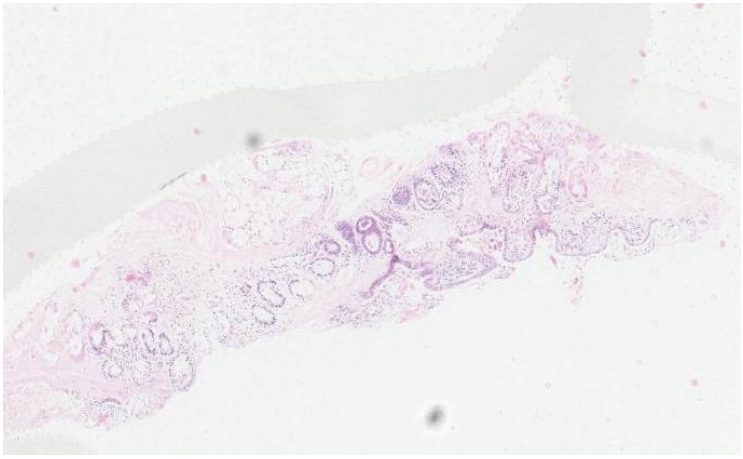
Ovalbumin 1mg/ml magnification x2.5



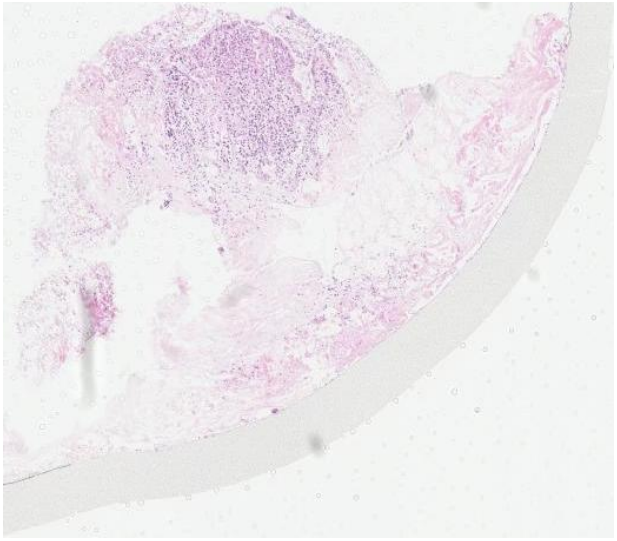
PT gluten 1mg/ml magnification x2.5



HMW glut04 200µg/ml magnification x2.5

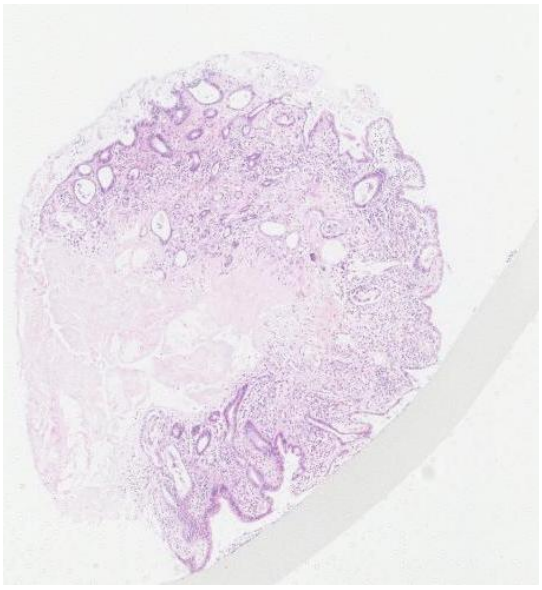


LMW glt156 200µg/ml magnification x2.5

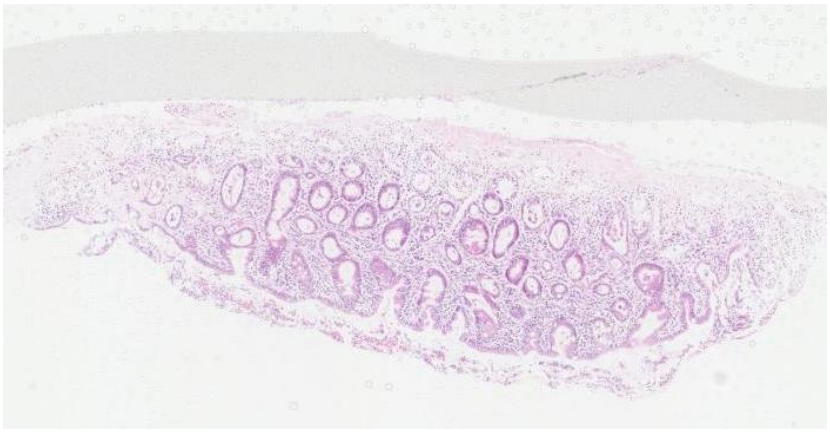


SM8: Refractory coeliac patient HLA DQ8 coeliac disease for 7 years and reftactory coeliac disease for 3 years

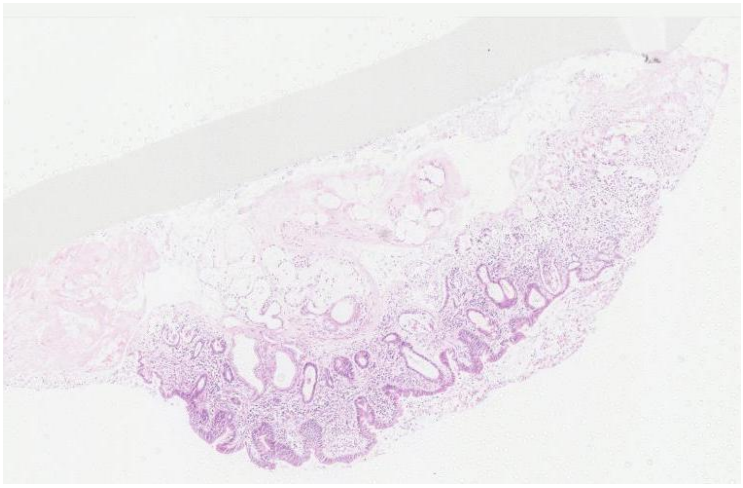
Medium-only magnification x2.5



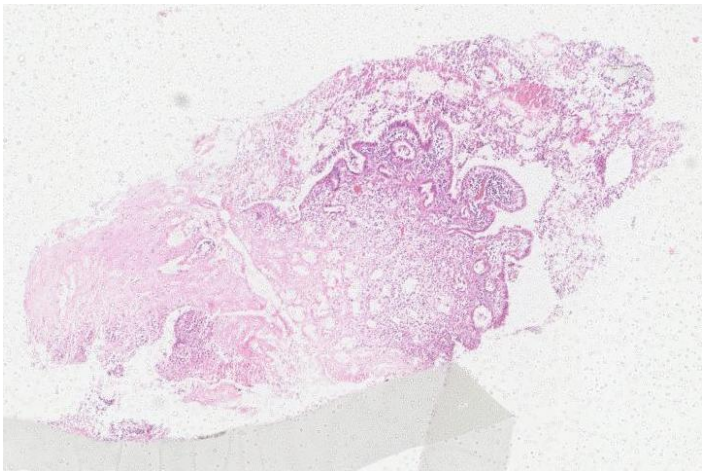
Ovalbumin 1mg/ml magnification x2.5



PT gluten 1mg/ml magnification x2.5



HMW glut04 200µg/ml magnification x2.5



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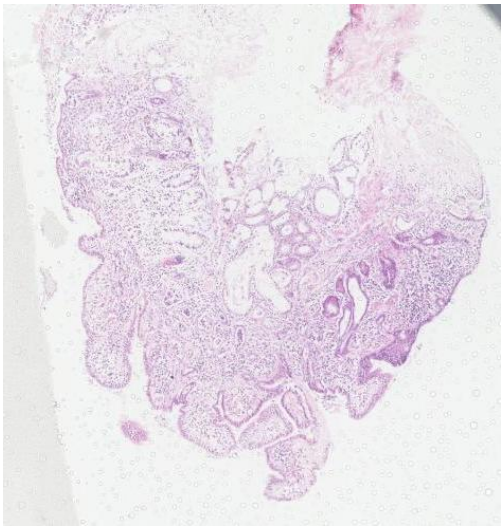
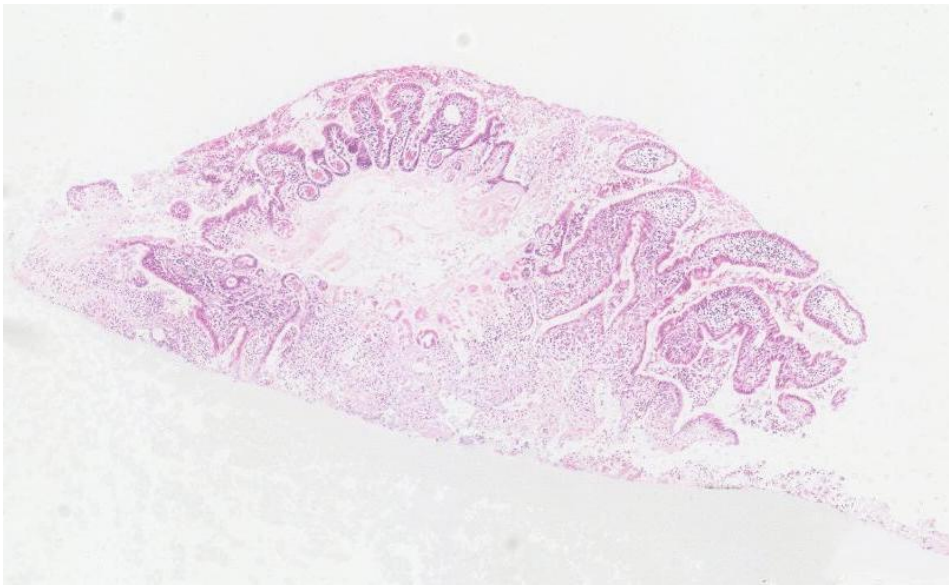


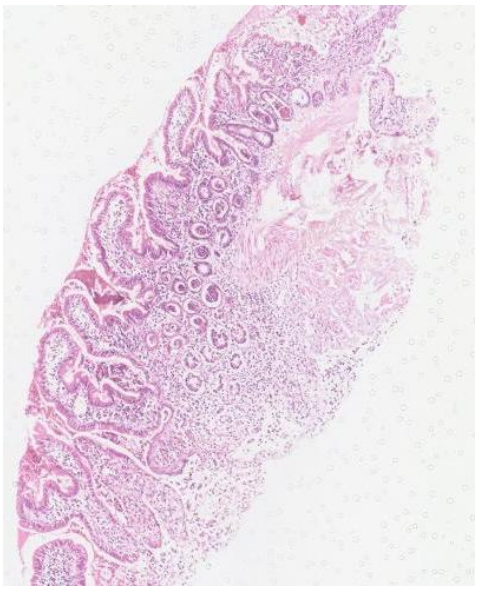
Control group of non-coeliac patients

SM14: investigation of IBS not HLA DQ2 or 8 positive

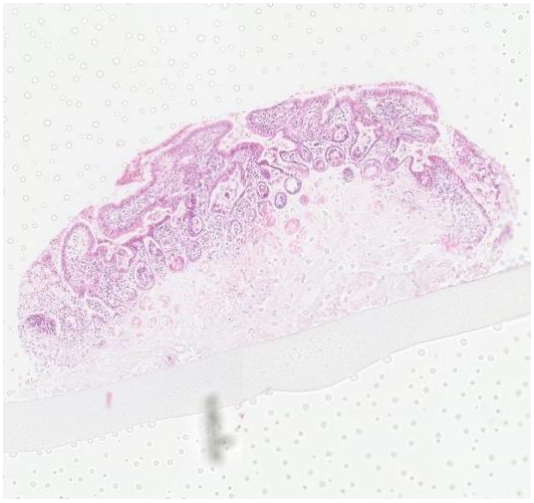
Medium-only magnification x2.5



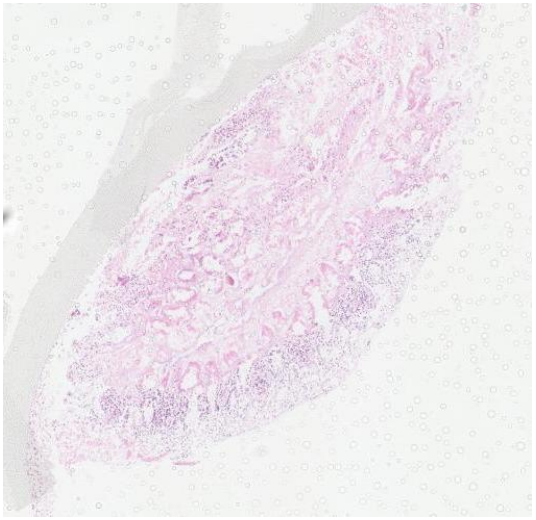
Ovalbumin 1mg/ml magnification x2.5



PT gluten 1mg/ml magnification x2.5



HMW glut04 200µg/ml magnification x2.5



LMW glt156 200µg/ml magnification x2.5